Sesquiterpene Lactones and Inulin from Chicory Roots: Extraction, Identification, Enzymatic Release and Sensory Analysis

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op vrijdag 20 maart 1992
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Stellingen

1 Het doen van sensorisch onderzoek naar de bitterheid van witlofwortel extract, verkregen door extractie met water, waarbij naar de bitterstoffen lactucine en lactucopicrine wordt gerefereerd, is zonder enige chemische analyse een hachelijke zaak.

Dit proefschrift.

Voirol, E. et al., 1987. Sensory analysis of chicory bitterness. In Martens, M., Dalen, G.A. & Russwurm Jr, H. (Eds.) Flavour Science and Technology. John Wiley & Sons, Chicester, p. 341-345.

2 Bij de sensorische analyse van witlof wordt te weinig rekening gehouden met de hydrolyse van sesquiterpeenlactonglycosiden door endogene witlofenzymen tot hun bittere aglyconen en een suikerrest.

Price, K.R. et al., 1990. Relationship between chemical and sensory properties of exotic salad crops - coloured lettuce (Lactuca sativa L.) and chicory (Cichorium intybus L.). J. Sci. Food Agric., 53 (2) 185-192.

Dirinck, P. et al., 1985. Objective measurement of bitterness in chicory heads (Cichorium intybus L.). In Baltes, W. et al. (Eds.) Strategies in Food Quality assurance: analytical, Industrial and Legal Aspects. De Sikkel, Antwerpen, p: III, 62-68.

3 Er kunnen vraagtekens worden gezet bij de vergelijking van een chemische analyse van bitterstoffen van een gekookt witlofmonster met de sensorische analyse van het equivalente niet gekookte monster.

Van der Meer, M.A. et al., 1985. De bitterheid van witloof. Proc. 8th International Biennal on chicory, 6 september, 1985, Geneve- Vaud, België.

4 Door het toevoegen van de titel van een referentie in de literatuurlijst van een publikatie, wordt de waarde van die referentie vergroot.

5 Halfvolle en vetarme levensmiddelen anders dan in melkproducten worden door de consument niet voor vol aangezien.

6 Het propageren van regels ten aanzien van gezonde voeding en het naleven ervan valt ook bij voedingsvoorlichters niet altijd samen.
Voedingsmagazine, 4 (3) 22-23 (1991).

7 Het is verbazend dat planten met dezelfde inhoudsstoffen, zoals cichorei (*Cichorium intybus* L.) en gifsla (*Lactuca virosa* L.) in de volksgeneeskunst voor zeer verschillende werkingen worden aanbevolen.

8 Het toepassen van witlofwortelextract als gewasbeschermingsmiddel tegen luizen dient in overweging te worden genomen, aangezien *L. virosa*, dat een hoog gehalte aan bittere sesquiterpeen lactonen heeft, niet door luizen wordt aangetast.
Eenink, A.H. & Dieleman, F.L., 1982. Resistance of Lactuca accessions to leaf aphids: components of resistance and exploitation of wild Lactuca species as source of resistance. Proc. 5th int. Symp. Insect-Plant Relationships, Wageningen. Pudoc, Wageningen, p. 349-355.

9 Research aan glycosidisch gebonden aromastoffen in planten zou niet alleen gericht moeten zijn op de vluchtige componenten, maar ook op alle niet vluchtige verbindingen.

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ABBREVIATIONS

dH8dLc	11(S),13-dihydro-8-deoxylactucin
dHLc	11(S),13-dihydrolactucin
dHLp	11(S),13-dihydrolactucopicrin
8dLc	8-deoxylactucin
Lc	lactucin
Lp	lactucopicrin
BuOH	butanol
CQA	caffeoylquinic acid
EtOAc	ethyl acetate
Glc	glucose
H_2O	water
HMF	5-hydroxymethylfurfural
KCN	potassium cyanide
MeOH	methanol
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
PCT	phenylthiocarbamide
PNPG	p-nitrophenyl-ß-D-glucopyranoside
tBuMeO	*tert*-butyl methyl ether
DP	degree of polymerisation
GC	gas chromatography
HPLC	high pressure liquid chromatography
m.p.	melting point
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance
RI	refractive index
RP	reversed phase
TLC	thin layer chromatography
UV	ultra violet

CHAPTER 1

INTRODUCTION

1.1 Historical background

Chicory (*Cichorium intybus* L.) is one of the many species of the Compositae family. It is a relative of the endive (*C. endivia* L.), the leaves of which are used as a vegetable, and the wild lettuce (*Lactuca virosa* L.). Chicory has been cultivated for the production of chicory leaves since approximately 300 BC. In classical antiquity roots as well as leaves of wild chicory were used in salads and as a vegetable. Aristophanes (450-380 BC) and Theophrastus (372-287 BC) mention the chicory plant and its use. Both Dioscorides and the elder Plinius discuss the healing power of chicory. It was said that the leaves and roots were stomachic and the juice was used against eye disease and poisoning. Infusions of the plant are said to be healthy for the liver, kidneys, and stomach. All of its parts (leaves, stems, flowers, seeds, and roots) have been employed as infusions for pharmaceutical purposes (Maier, 1987; Schmiedeberg, 1912).
During the Middle Ages the Arabs used chicory as a remedy and foodstuff, and in Europe it was used as a medicine, and as a magic potion (Maier, 1987).
It is not known when chicory was roasted for the first time for use as coffee substitute. Coffee-like brews made from chicory were used in Italy and Belgium in the 16th century. In 1592 Prosperus Alpinus described such beverages. In 1690, the beverage is mentioned in Holland, and it seems that it was used as a coffee substitute. In the 17th century the use of coffee became quite common in Europe, and simultaneously also the use of chicory roots as a coffee substitute increased (Maier, 1987; Schmiedeberg, 1912).

At the end of the 18th century large plantations of chicory had been established in France and Prussia. The first factory for roasting chicory was founded in Holzminden, Germany. During the Continental System (1806-1813)

almost no coffee was imported into Europe. As a consequence the cultivation of chicory and the use of the roasted root as coffee substitute spread over the continent.
In 1882 more than 125000 tons of roasted chicory were produced in Europe. The annual world production of fresh roots during the first decade of this century for roasting purposes was estimated at 700000 t. In 1985 the world-wide consumption of dried roots was approximately 128000 t (ca. 450000 t fresh roots). Of this amount 57% was consumed in Western Europe, 20% in Eastern Europe, 2% in the United States, and the rest in South Africa and India (Maier, 1987).

Nowadays the consumption of roasted chicory as a coffee substitute in the Netherlands is practically limited to vegetarians. Today chicory leaves (Belgian endive, witloof chicory) are cultivated for vegetable consumption, and the roots constitute a waste product. Chicory is no longer used for major pharmaceutical purposes, but may still constitute a folk medicine (Cecchini, 1976).

Chicory may also be used as a raw material for the alcohol industry (Saryanov et al., 1938), because of its inulin content, the main polysaccharide of chicory roots. The advantages of chicory over potatoes as a raw material in the alcohol industry was critically evaluated by Garger (1938). In 1986 a patent was filed in which a procedure is described for liberation of fermentable sugars present in chicory roots by an enzymatic process. These sugars may be used for alcoholic fermentation (de Baynast de Septfontaines, 1986).
Gupta and co-workers (1988, 1989) used an aqueous extract of chicory root as starting material for the production of inulinase (β-fructofuranosidase, EC-3.2.1.7) and fructose.
Chicory is also used as ingredient for the preparation of soft drinks (Leroux, 1987b; Vonasek et al., 1986; Stat. Cultura Sfecle, 1985). Eres'ko et al. (1987) prepared condensed milk with chicory. Nachmedov & Kuljasova (1988) defined a juice made from a chicory-apple mixture as a coffee substitute, but according to their description it is more like a soft drink.

1.2 Production of chicory

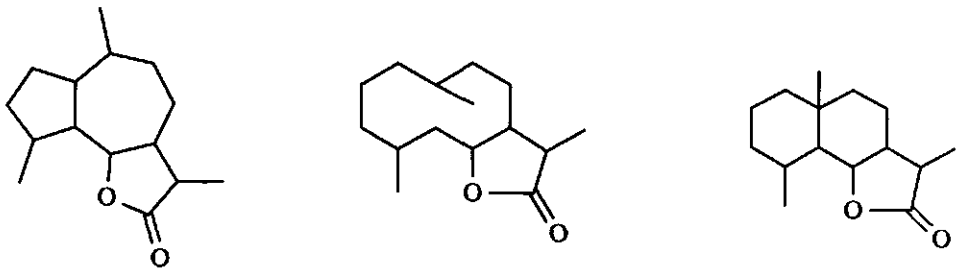
Chicory is cultivated for the production of chicory leaves or heads (Belgian endive, witloof chicory) for use as a vegetable. It is appreciated because of its fine, slightly bitter taste. The production of witloof is mainly concentrated in Northern Europe.
Production comprises of two phases, the cultivation of the roots in the field, and the forcing of the heads (chicons) in so-called trenches or pits (Huyskes, 1962). In the Netherlands the seed is sown in May or early June, and the carrot-like roots are harvested from late September to the end of November. After removing the foliage, the roots are embedded in pits. They are placed very close together in soil of good structure and provided with ample supplies of water. The roots are covered with a layer of soil. After 10 or more days a heating system underneath the roots is turned on. A few weeks later the well-known white heads are being produced.
At harvest the root and head are taken out of the pit in their entirety. The heads are cut off, cleaned, graded, and packed.
The roots are considered as waste. In the Netherlands about 10^5 tons of chicory roots are produced annually. They may be used as cattle feed. However, they contain both the valuable fructose polymer inulin and bitter principles.

The food industry is interested in inulin and new bitter compounds, such as sesquiterpene lactones from chicory. Additionally there is a demand in the Netherlands for the evaluation of new crops to incorporate into the Dutch cropping plan (Koster & Schneider, 1989) due to stagnation on agricultural markets in the food sector, and problems associated with intensive agricultural production.
According to Fuchs (1989) a few crops, among them chicory and Jerusalem artichoke, have been considered for introduction. Both of these are inulin containing crops, and can be used for the production of high-fructose syrup as sweetener, or as raw material for the production of 5-hydroxymethylfurfural (HMF). HMF can be used as starting material for the synthesis of many other products, among them dyes, flavours, and antibiotics (Kuster, 1989).
Recent data suggest that chicory can give higher inulin production compared

with Jerusalem artichoke, and that chicory can be produced for the same price as sugar beets when the more intense sweetness of fructose is taken into account (Meyer & Borm, 1990).

1.3 Specific constituents of chicory roots

The bitter compounds isolated from chicory roots are sesquiterpene lactones with a guaiane skeleton (Fig. 1.1) (Leclercq, 1984; Pyrek, 1985; van Beek et al., 1990). Seto et al. (1988) identified also sesquiterpene lactones with a germacrane and eudesmane skeleton in chicory roots (Fig. 1.1), but until now these compounds have not been evaluated for their probably bitter taste.



guaiane germacrane eudesmane

Fig. 1.1 Structural formulas of types of sesquiterpene lactone skeletons identified in chicory root.

Chicory, like other members of the Compositae family, is distinctive in containing inulin (fructan, fructosan) as the main carbohydrate. Inulin is a fructose polymer with a Degree of Polymerisation (DP) in excess of 30 (McKellar & Modler, 1989). The molecule is terminated by a glucose molecule (Fig. 2.6). Inulin may be used as raw material for fructose production. Fructose is 1.3-1.6 times sweeter than saccharose (Hyvönen et al., 1977) and can thus help to reduce the caloric value of the diet. It seems also to be less cariogenic when compared with saccharose.

Inulin as such can not be digested by human beings due to the absence of the appropriate enzymes. *Bifidobacterium* sp. are present in the intestine, which have inulinases that can only hydrolyse neosugars, fructosans with a DP of 3-6 and not fructosans with higher DP (McKellar & Modler, 1989).

In addition to the bitter taste and the presence of inulin, brews made from roasted chicory roots have coffee-like characteristics. Such brews have been consumed in Western Europe for almost as long as coffee itself. They have generally been and still are regarded as substitutes for coffee. This is especially true in times of restricted supplies (Clarke & Macrae, 1987).
A brew made from roasted chicory does not contain any caffeine as a stimulant. This lack of caffeine is considered to be an advantage to particular consumer groups, for example vegetarians. Chicory has also been attributed with other physiological benefits, among others stimulating the appetite, and aiding the digestion (Leroux, 1987a). The constituents of chicory roots will be discussed with more detail in Chapter 2.

1.4 Processing of vegetables

Chicory roots are considered a waste. They are produced at the harvest of the edible chicory leaves. The roots contain inulin as well as bitter sesquiterpene lactones. As a very cheap raw material it may be used for isolation of both types of compounds in order to obtain a bitter, sweet liquid. This liquid might be of use as an ingredient in alcoholic and non-alcoholic beverages. The bitter compound quinine could be replaced by the bitter principles from chicory roots. Quinine is a pharmaceutical product used against malaria and should therefore not be applied to food products. Some people suffer from adverse reactions when quinine is included in the diet.
As part of the Biotechnology Research Progamme of the Ministry of Agriculture and Fisheries, a project was formulated to isolate both the bitter compounds and inulin from chicory roots with the use of enzymes and/or starter cultures.
Usually the isolation of interesting compounds from plant material is carried out with a solvent. Simultaneous extraction of both bitter substances and

inulin from chicory roots with one solvent is not possible. Inulin is extracted with (hot) water using countercurrent diffusion. The sesquiterpene lactones are rather apolar and will therefore remain in the pulp. Extraction with a more apolar solvent gives only the bitter compounds as solvent solubles, and inulin remains in the pulp fraction. This waste material will also contain a large amount of organic solvents. To reduce the inherent risks, general safety and environmental measures would have to be taken, which induce extra costs.

Enzymatic liquefaction of plant material will release all its substances. The amount of waste material is reduced, and the liquid phase contains all interesting compounds. A liquid is easy to handle during processing.

The application of enzymes is common in the fruit industry (see below). The possible use of enzymes in vegetable processing by various groups was evaluated.

For instance sauerkraut is the product of a lactic acid bacterial fermentation of cabbage. It is made by adding salt to shredded cabbage, followed by packing the salted cabbage into containers allowing subsequent fermentation. Other vegetables are also known to undergo fermentation processes, e.g. cucumbers, olives, and on a smaller scale cauliflowers, carrots, beans, celery, and onions. Lactic acid fermentation of vegetables is described in more detail by Pederson (1980) and Vaughn (1982).

The application of enzymes in fruit processing originates from the use of pectolytic enzymes for treatment of soft fruit to ensure high yields of juice and pigments and for the clarification of raw press juices. Technical enzyme preparations in use for these processes have mainly pectolytic activities along with other enzymes such as xylanases and arabinases (Voragen, 1989).

Press juices obtained from for instance apples, are rather viscous due to dissolved pectin and have persistent turbidity, because of cloud particles. Addition of a pectinase preparation to the juice decreases viscosity and also degrades the negatively charged pectin coating of the suspended particles. As a consequence destabilised particles coagulate to larger units and precipitate (Voragen, 1989). A centrifugation and/or filtration step to remove these particles is necessary to obtain a clear juice, in many cases after addition of gelatin to remove polyphenols.

Pectinases are also used for the preparation of vegetable juice, especially carrot juice. Juice yield as well as the carotene content of the juice

increase when pectinases are involved (Leclercq, 1985).
When enzymatic treatment of fruit or vegetable pulp with pectolytic enzymes is carried out together with cellulolytic enzymes, an almost complete liquefaction can be obtained. This process reduces solid waste and seems especially suited for products for which no juice extraction equipment has been developed, e.g. tropical fruit (Voragen, 1989). Yield of the obtained juice increases up to 90%, and almost all dry matter content is found in the liquid phase. In theory the whole cell content is present in the juice, which otherwise would be lost with the pulp fraction. Only a few insoluble residue particles remain (Pilnik & Rombouts, 1979).

Enzymatic liquefaction is an unusual process for isolation and extraction of flavour compounds from plant material. Only a few publications are known in which the use of enzymes ((hemi)cellulases) has been applied in order to increase the yield of aromatic or antimicrobial compounds (e.g. Tateo and co-workers, 1979, 1977, 1982; Chuyen et al., 1982; Szakács-Dobozi et al., 1988). Nitz et al. (1985) incubated *Majorana hortensis* first with pectinase or glucosidase preparations before extraction by steam distillation under reduced pressure. They compared these extracts with those from untreated plant material. Enzymatic treatment increased the yield of flavour compounds and affected the composition of the extracts when compared with the control. They suggested that bound precursors are present in the plant, which may be transformed to different components (aglycons) depending on the extraction method used.

Flavour compounds in the plant are present in the cell in free form, and as glycosidic non-volatile precursors which are rather polar (see e.g. Wilson et al., 1984). Beta-glucosidases might be used for the hydrolysis of monoterpene glycosides to enhance the aroma of grape juices and wines through the release of volatile monoterpenes (Voragen, 1989). The same mechanism might be expected for sesquiterpene lactones glycosides, which were isolated from chicory roots for the first time by Seto et al. (1988). These enzymes are present in commercial enzyme preparations containing cellulolytic activities.

1.5. Outline of this study

As a part of the Biotechnology Research Programme of the Dutch Government (Four Million Guilder Fund) we planned to isolate bitter substances as well as inulin from chicory roots in a one step enzyme treatment using a commercial enzyme preparation containing both pectinases and cellulases to obtain complete liquefaction of the roots. As a consequence more bitter compounds and sugars (inulin) would pass into the liquid phase. No second extraction step would be required, and no heat treatment involved, which might otherwise alter the amount of isolated compounds and give undesirable off-flavours. The completely liquefied root suspension can be used as raw material in the manufacture of soft drinks.
As a consequence analytical methods had to be improved, and studies on isolation, identification, and last but not least on the bitterness of sesquiterpene lactones were carried out.

The results of experiments presented in this thesis were obtained in the various laboratories involved in this project. The project was started at the former Sprenger Institute (now part of ATO Agrotechnologie), Wageningen, in co-operation with the Agricultural University, Wageningen, Section Food Chemistry. For identification of the bitter compounds we could rely on the experience of scientists from the Department of Organic Chemistry of the same University. During the second part of the project the co-operation with Organic Chemistry intensified, and also Quest International became involved with respect to sensory analysis.
During a part of the project a lively exchange of ideas with Prof. Gensch, Freie Universität, Berlin, working in the same field, took place.

CHAPTER 2

THE COMPOSITION OF CHICORY

2.1 Fresh chicory roots

2.1.1 *Sesquiterpene lactones*

Schmiedeberg (1912) isolated the bitter principle from chicory roots, a compound he named intybin. Grafe (1915) found that intybin was neither an alkaloid, nor chlorogenic acid or tannin, and that by roasting the bitter compounds were destroyed. Grafe showed intybin to be a glycoside with fructose, and not toxic to animals. However, experiments with animals were not clearly described.
Grafe (1915) did not structurally characterise the intybin, because his material was not pure. However, in 1936 he determined an empirical formula: $C_3H_4O_2$ (Grafe, 1936). He also found that intybin was composed of 25% proto catechuic aldehyde and 75% inulin, the main polysaccharide present in chicory root. When inulin was included in the calculation Grafe found a molecular formula $C_{24}H_{32}O_{16}$. Probably the isolated compound was not pure enough, because both formulas subsequently have been proved false. Earlier Zellner & Richling (1926) found that intybin is identical with lactucin.

Lactucin is also one of the components found in lactucarium, the dried milky juice of *Lactuca virosa* L. (Schenck & Graf, 1936). It was isolated from lactucarium by extraction with water. Lactucin as bitter compound was described for the first time by Buchner in 1833 (Schenck & Graf, 1936). A second bitter tasting compound in lactucarium, lactucopicrin, was mentioned by H. Ludwig & A. Kromayer (Arch. Pharm., 161, 1-3 (1862), cited by Schenck & Graf, 1936). They did not show which type of compound lactucopicrin was nor did they give the molecular formula. For lactucin two formulas were given: $C_{23}H_{14}O_8$ and $C_{22}H_{13}O_7$, but both are incorrect.

Lactucarium had the interest of scientists because of its potential pharmaceutical properties. Lactucarium has been used as a hypnotic and for sedative purposes. It tasted very bitter, as does its aqueous extract. In the 19th century it was proposed that the bitter compound of lactucarium was responsible for its sedative properties (Schenck & Graf, 1936). Much attention was therefore given to identification of the bitter (and possibly sedative) component of lactucarium.

Bauer & Schub (1929) and Bauer & Brunner (1936, 1937, 1938) identified in lactucarium in addition to lactucin also lactucerol ($C_{30}H_{49}OH$) and lactucerin, which are resinous compounds, and neolactucin ($C_{23}H_{25}O_7$, m.p. 147-148 °C) in the fresh latex of *Lactuca virosa*. Two enzymes (oxidases) were also identified. The primary bitter compound of lactucarium and of the fresh milky juice of *L. virosa* was named neolactucin. Späth et al. (1951) suggested that neolactucin is the same component as lactucopicrin. No further work has been reported to identify the resinous compounds of lactucarium mentioned by Bauer and co-workers.

In 1939 Späth et al. published an extraction method to isolate lactucin from lactucarium with diethyl ether. It is a rather difficult method, and time consuming, but pure lactucin (m.p. 220-223 °C) was obtained.

After World War II publications on lactucarium concern only the identification of lactucin, lactucopicrin and other sesquiterpene lactones from *Lactuca* species. No publications are known in which the relationship between the sedative properties and bitter principles of lactucarium is establised.

Späth et al. (1951) isolated pure lactucin from lactucarium and obtained the correct molecular formula: $C_{15}H_{16}O_5$. Previously published formulas of lactucin have been incorrect, because of the low solubility of lactucin (Späth et al. 1951). They argued that different methods for determination of the molecular weight of lactucin give variable results, because several types of solvents were used.

Wessely et al. (1951) proved that lactucin has an unsaturated character with an absorption maximum at 255 nm ($\log \epsilon$=4.17). They did not mention the solvent in which lactucin was dissolved for UV measurement.

In 1940 Schmidt showed that Compositae other than *L. virosa* contained lactucin and/or lactucopicrin, including *Cichorium intybus* L., chicory. A

quantitative method for determination of lactucin and lactucopicrin from the latex of *L. virosa* was published (Schmidt, 1940). Lactucin and lactucopicrin were determined after reaction with potassium cyanide (KCN) dissolved in methanolic NaOH in light (photochemical reaction) or in dark (so-called fluorescence reaction). After reaction with KCN in light a yellow product was obtained from lactucin (Lc) and a red product from lactucopicrin (Lp). Both products were measured by UV spectroscopy. The reaction of Lc and Lp with KCN in dark gave products with an intensive blue fluorescence character. No further details were given on the structure of the obtained products. The cyanide ion is able to add to (α,β-unsaturated) carbonyl groups not only from Lc and Lp, but also from other compounds present in the extract.

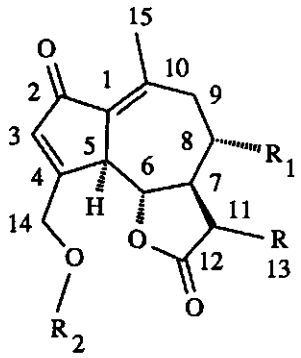
Zinke & Holzer (1953) and Holzer & Zinke (1953) isolated lactucin as well as lactucopicrin from the milky juice of chicory. They showed lactucopicrin to be the same as neolactucin, and determined the molecular formula as $C_{23}H_{22}O_7$. A lactone ring was shown to be present in both compounds. Lactucopicrin (m.p. 148-151 °C) is the p-hydroxyphenyl acetic acid ester of lactucin (m.p. 220-223 °C) (Zinke & Holzer, 1953).

Dolejs et al. (1958) and Barton & Narayanan (1958) published independently the structure and absolute configuration of lactucin (Fig. 2.1). They proved it to be a sesquiterpene lactone with a guaianolide skeleton and with the unsaturated lactone group attached to the 6-position. One of the two hydroxyl groups is secondary; it is in the 8-position. It is the first sesquiterpene lactone of the azulene series with a carbonyl group at C-2 (Barton & Narayanan, 1958).

In 1960 Michl & Högenauer published the chemical structure of lactucopicrin. They thought that the p-hydroxyphenyl acetic acid part of lactucopicrin is esterified to lactucin at C-14. However, Pyrek (1977) proved with NMR, IR, and MS that the site of esterification of lactucopicrin with p-hydroxyphenyl acetic acid is at C-8 in the lactucin molecule (Fig. 2.1).

A third sesquiterpene lactone identified in chicory was 8-deoxylactucin (8dLc)(Leclercq, 1984; Pyrek, 1985). This compound was also detected in *L. serriola* (Pyrek, 1977). The secondary hydroxyl group at C-8 is reduced compared with Lc (Fig. 2.1).

In 1988 Japanese scientists (Seto et al., 1988) isolated four new components together with known sesquiterpene lactones from *Cichorium intybus* and *C. endivia* roots. The following compounds were identified in *C. intybus*: 8-



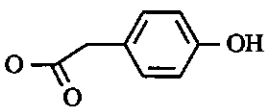
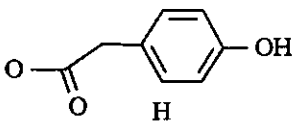
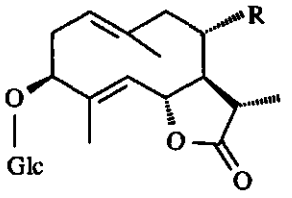
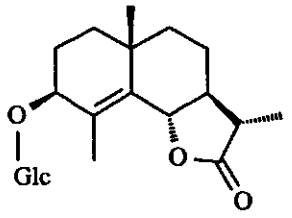
	R	R_1	R_2
Lc	$=CH_2$	OH	H
8dLc	$=CH_2$	H	H
Lp	$=CH_2$		H
dHLc	$\cdots CH_3$	OH	H
dH8dLc	$\cdots CH_3$	H	H
dHLp	$\cdots CH_3$		H
Crepidiaside B	$\cdots CH_3$	H	Glc
Cichorioside B	$\cdots CH_3$	OH	Glc

Fig. 2.1 Sesquiterpene lactones with a guaiane skeleton isolated from chicory roots.

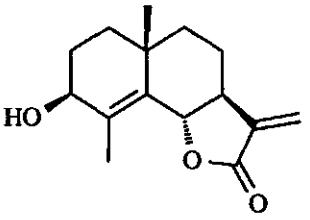


Sonchuside A R= H

Cichorioside C R= OH



Sonchuside C



Cichoriolide A

Fig. 2.2 Sesquiterpene lactones with an eudesmane and a germacrane skeleton isolated from chicory roots.

deoxylactucin, crepidiaside B (glycoside of 11(S),13-dihydro-8-deoxylactucin (syn. jacquilenin)), and cichorioside B (glycoside of 11(S),13-dihydrolactucin), all having a guaiane skeleton (Fig. 2.1); sonchuside A, and cichorioside C, having a germacrane skeleton (Fig. 2.2); and sonchuside C, and cichoriolide A, having a eudesmane skeleton (Fig. 2.2). Surprisingly they did not identify lactucin and lactucopicrin in chicory.

Van Beek et al. (1990) isolated lactucin, 8-deoxylactucin and lactucopicrin from chicory roots, together with their respective 11(S),13-dihydro derivatives. The 11(S),13-dihydrolactucopicrin was isolated and identified

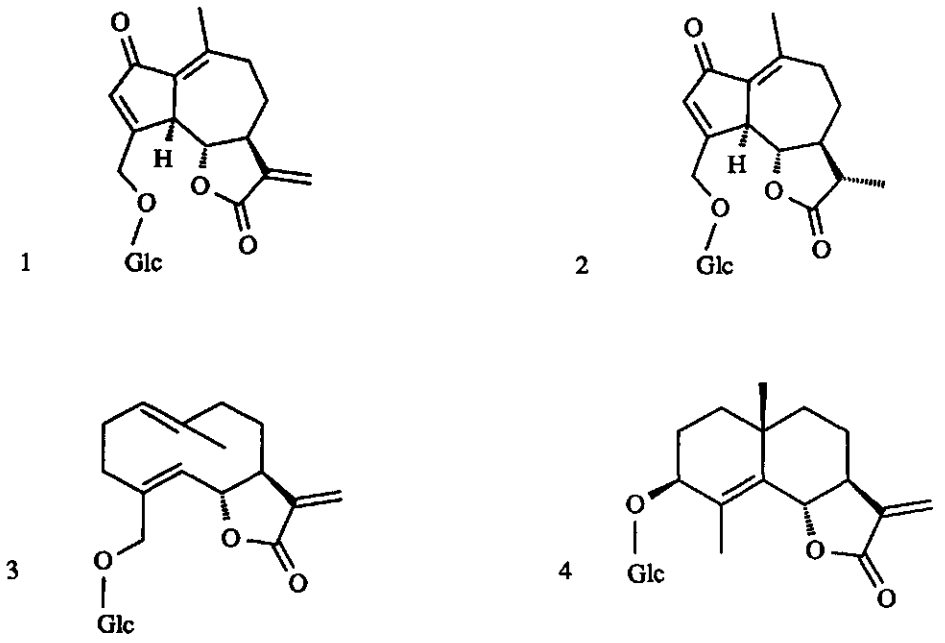
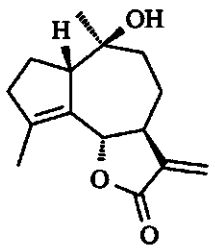
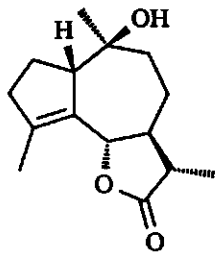


Fig. 2.3 Sesquiterpene lactones isolated from Cichorium endivia (after Seto et al., 1988).
1=crepidiaside A; 2=crepidiaside B; 3=picriside B; 4=cichorioside A.

from *C. intybus* for the first time (Fig. 2.1).
Related sesquiterpene lactones were isolated and identified from *Cichorium endivia* L. and *C. pumilum* Jacq. (Seto et al., 1988; El-Masry et al., 1984; see Fig. 2.3 and 2.4 respectively).
Lactucin and lactucopicrin are, according to Schenck & Graf (1936) responsible for the bitter taste of lactucarium. Both compounds have been identified in chicory (Schmidt, 1940). They are held responsible for its bitter taste. However, analytical methods have become more sensitive enabling more components to be detected in chicory root. Van Beek et al. (1990) reported for the first time that 8-deoxylactucin and the 11(S),13-dihydro derivatives of lactucin, 8-deoxylactucin, and lactucopicrin also taste



1



2

Fig. 2.4 Sesquiterpene lactones isolated from Cichorium pumilum (after El-Masry et al., 1984).
1=10(S)-hydroxycichopumilide; 2=10(S)-hydroxy-11(S),13-dihydrocichopumilide.

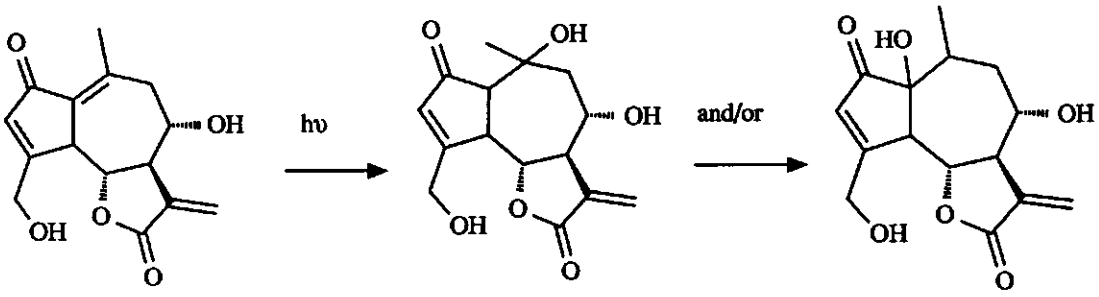


Fig. 2.5 Proposed reaction scheme for the addition of water to lactucin (after Schenck et al., 1964).

bitter.

Schenck et al. (1953, 1963, 1964) reported that lactucin and lactucopicrin, when dissolved in water, are not stable in light. Under the influence of daylight, addition of water to lactucin and lactucopicrin occurs. Schenck and co-workers (1964) gave an explanation for this reaction (Fig. 2.5).

Leclercq et al. (1988) proved that 8-deoxylactucin is also not stable in daylight when dissolved in water. However the end products were not analysed or tasted for bitterness.

Lactucopicrin as well as 8-deoxylactucin proved to be an antifeedant for the locust (*Schistocerca gregoria*) at levels comparable to those present in chicory (see also Chapter 2.1.3.7)(Rees & Harborne, 1985).

2.1.2 *Carbohydrates*

The main carbohydrate in chicory roots is inulin, a linear β-(2-1) linked fructose polymer terminated by a sucrose unit residue (Fig. 2.6). Its average molecular weight is about 6000 Daltons (35 DP). Inulin is only slightly soluble in water at 15 °C (225 mg/100 ml), but more so at 50 °C (1737 mg/100

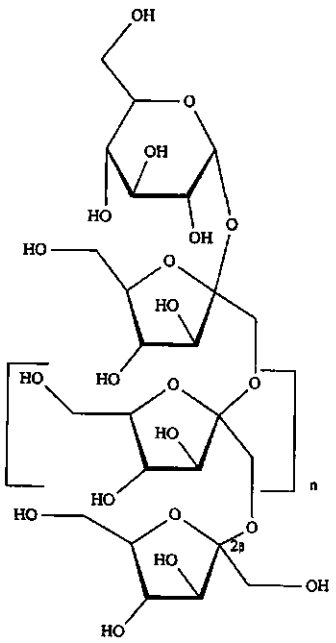


Fig. 2.6 Inulin.

ml)(Yanovsky & Kingsbury, 1933). The inulin content of chicory roots is approx. 16% (w/w), but the exact percentage depends on storage conditions.
Inulin can be used as raw material for fructose production. Fructose is an interesting sweetener. The sweetness and functionality of fructose syrups are considered satisfactory for complete or partial replacement of sucrose (Zittan, 1981).
The use of inulins as raw material is connected with their molecular weight distribution. For the production of fructose long-chain inulins are advantageous because after hydrolysis only little of the crystallization inhibitor glucose is obtained. Short-chain oligosaccharides are easily fermented by microorganisms, and thus are favourable for fermentation (Beck & Praznik, 1986).
Gupta and co-workers (1986) elucidated the fructosan metabolism in chicory roots. Different fructosyl transferases seem to be responsible for the various fructosylation steps in fructosan (inulin) synthesis.

Inulin is hydrolysed during storage of chicory roots. The extent of hydrolysis depends on time and temperature. After 5-6 weeks of storage at 3 °C, 90% of insoluble inulin (DP>10) is hydrolysed by an inulin degrading enzyme, increasing the soluble sugars (DP<10) (Rutherford & Jackson, 1965). These results have been confirmed by Rutherford & Weston (1967) and Fiala & Jolivet (1980).
Forcing of the chicory roots also influences its carbohydrate content . Total carbohydrate content decreases considerably whilst the amount of reducing sugars increases. The duration of cold storage of the roots is found to have little effect on the carbohydrate changes which occur during forcing (Rutherford & Phillips, 1975).

Extraction of inulin from plant material is possible by diffusion at elevated temperatures, or by enzymatic liquefaction (Chapter 7). Besides chicory roots, Jerusalem artichoke and dahlia can also be used for inulin production (Praznik & Beck, 1986; Fleming & Groot Wassink, 1975).
Inulin can be hydrolysed by acid under relatively mild conditions: pH 1 to 2 for 1-2 h at 80-100 °C (Zittan, 1981). Since fructose is easily degraded by acid through the processes of enolization and dehydration, flavoured and coloured compounds are produced which are undesirable in a syrup. Therefore

the pH, temperature, and time must be adjusted to minimize fructose degradation (Fleming & Groot Wassink, 1975).
Several enzymes capable of hydrolysing inulin have been described in the literature. Microbial enzymes originate mainly from yeasts and moulds, for example *Kluyveromyces fragilis*, *Aspergillus* sp. (Zittan, 1981). Complete enzymatic hydrolysis of inulin could be obtained at 50 °C in 3 h. Colour and flavour changes in the enzyme hydrolysate were minimal. So enzymatic treatment of inulin for fructose production is preferable to hydrolysis by acid (Fleming & Groot Wassink, 1979).
Gupta and co-workers (1988, 1989) studied the possibilities for using an aqueous extract of chicory roots containing fructosan (inulin) as raw material for fructose and inulinase production. They cultivated *Fusarium oxysporum* on a medium prepared from chicory roots, and the produced inulinases from *F. oxysporum* hydrolysed inulin giving primarily fructose.

Other carbohydrates present in chicory roots, besides sucrose, fructose and some glucose (Pazola, 1987), are cellulose, hemicelluloses, and pectin. These components provide the structural skeleton of both individual plant cells and the plant as a whole, unlike inulin, which is a storage carbohydrate. Pazola (1987) and Leroux (1987a) mentioned a crude fiber (cellulose) content of chicory roots of 5% and 4% (based on dry weight) respectively.
Hageman (1983) found a cellulose content of 3.4 to 5.5% in chicory roots dependent on forcing of the roots. Pectin content varied from 6 to 10%. All data are expressed as percentage of dry weight. Kim et al. (1978) found in dried chicory root 6.9% "crude fiber".

2.1.3 *Other components*

2.1.3.1 Nitrogenous compounds

The amounts of nitrogenous compounds (free amino acids and proteins) in chicory roots are not very high. Pazola (1987) mentioned a total nitrogen content of 6-9% (dry weight), which was confirmed by Leroux (1987a). Kim et al. (1978) found for dried chicory roots a "crude protein" content of 8.6%.

2.1.3.2 Lipids

Lipids are present in chicory roots in small amounts, 0.1-0.2% (dry weight) (Pazola, 1987). A crude fat content of 1.6% based on dried chicory root was found by Kim et al. (1978). The amount of unsaturated fatty acid is 65.4% of the total fat of the roots, of which linoleic acid represents the main part. The essential oil (0.007%) obtained from air-dried chicory root consists of 60% palmitic acid, 31.5% linoleic acid, 2.9% linoleic acid, 1.8% n-pentadecanoic acid, and 0.9% oleic acid (Sannai et al., 1982).

2.1.3.3 Minerals

The ash content of chicory roots is 4-6% (dry weight) (Pazola, 1987). The most important mineral components are potassium, sodium, calcium, and magnesium. Kim et al. (1978) identified phosphorus, and iron in chicory roots, and traces of other mineral compounds (zinc, copper).

2.1.3.4 Organic acids

Recently Gaber & Maier (1989) analysed and identified the acids of the chicory roots using HPLC. Dried and roasted samples were analysed. The main acids found in the roots were citric (9.80-10.00 g/kg) and malic (8.96-9.56 g/kg). These are also mentioned by Pazola (1987), but the amounts are much lower. Other acids detected in chicory roots are: formic, acetic, quinic, lactic, hydroxyacetic, phosphoric and pyroglutamic acid.

2.1.3.5 Alkaloids

No alkaloids were isolated from fresh chicory roots contrary to roasted roots (see Section 2.2.3.5)

2.1.3.6 Chlorogenic acids

Chlorogenic acid is a polyphenolic compound, which is a substrate for phenolases. The brown pigments that develop on the cut surface of fruit and vegetables is caused by the activity of this enzyme.

The term chlorogenic acid is used for in fact a mixture of various caffeoylquinic acids (CQAs). In chicory are determined 3-CQA (Fig. 2.7), 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (Haffke & Engelhardt, 1986; Clifford et al., 1987).

The chlorogenic acid content of unroasted chicory root, as well as coffee

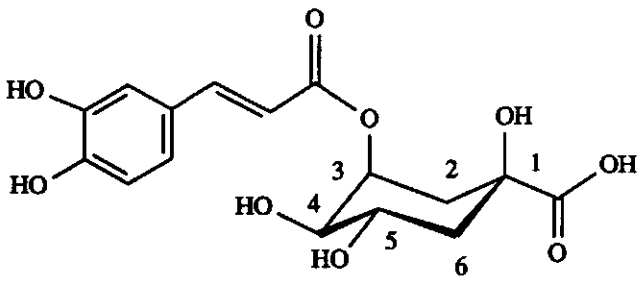


Fig. 2.7 Chlorogenic acid (3-caffeoylquinic acid, 3-CQA).

substitutes containing a mixture of roasted chicory and dandelion was determined by Clifford et al. (1987). Macrae et al. (1987) reported a total chlorogenic acids content of 3.5% in fresh chicory roots. This rather high value is probably due to the non specific spectrophotometric method used.
Determination of chlorogenic acid is one of the methods for quantitative analysis of the composition of coffee mixtures containing coffee substitutes. Coffee, in the form of roast beans or as instant powder, will contain significant amounts of chlorogenic acid. Most coffee substitutes do not contain appreciable levels of CQA. This provides the basis for a quantitative method for determining the proportion of coffee in mixtures (Macrae et al., 1987).

2.1.3.7 Coumarins

According to van Hee (1965) coumarins are also bitter. Already in 1876 Nietzki isolated a coumarin from the flowers of chicory, and named it cichoriin (Fig. 2.8 [3])(van Hee, 1965). Cichoriin is a glycoside of esculetin or dihydrocoumarin [1]. A third coumarin esculin [2], which is an isomer of cichoriin has been isolated from chicory. The three coumarins are mainly found in the leaves and flowers of chicory (Fedorin et al., 1974; Rees & Harborne, 1985). Only cichoriin is found to be present in the roots (Pazola, 1987; Rees & Harborne, 1986).
Rees & Harborne (1985) investigated coumarins isolated from chicory roots as antifeedant for locusts (*Schistocerca gregaria*). It was found that cichoriin

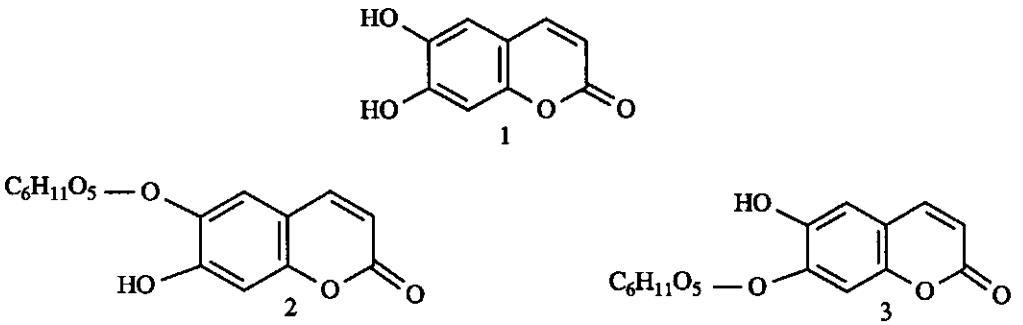


Fig. 2.8 Coumarins from chicory.
1=esculetin; 2=esculin; 3=cichoriin.

is still a significant antifeedant at low concentrations (0.006% (w/w)), while esculetin and esculin were inactive at this concentration.
According to Rees & Harborne (1985) these results are contrary to other findings. Coumarin itself inhibits feeding of some insect species only in relatively high concentrations (0.4% (w/w)). Hydroxycoumarins and/or their derivatives had no antifeedant properties (Rees & Harborne, 1985). However, these findings explain in part the recognized pest resistance of the chicory plant.

2.1.3.8 Volatile compounds

Until now no literature is known on volatile components of fresh unroasted chicory roots. Research on volatile compounds of chicory leaves has been carried out at the State University of Gent (Belgium)(P.J. Dirinck, pers. comm., 1990). Among other compounds n-hexanal and cis-3-hexenol were identified, which are responsible for the "fruity" and "green" flavour respectively.
Sannai et al. (1982) analysed the essential oil obtained from air-dried chicory roots. This oil consisted mainly of palmitic acid and linoic acid (see Section 2.1.3.2).

2.2 Roasted chicory roots

2.2.1 *Sesquiterpene lactones*

Roasting of chicory roots is carried out at 160-170 °C for 1-1½ h (Stoltze, 1987). Grafe (1915) could not isolate any bitter sesquiterpene lactones from the roasted roots. He assumed that during the roasting process these compounds are degraded. Also Pazola (1987) suggested that the natural bitter substances of chicory are destroyed during roasting. No recent literature was found which described the extraction and analysis of sesquiterpene lactones from roasted roots, and whether they are degraded during roasting or not.

2.2.2 *Carbohydrates*

The main constituents of chicory roots, i.e. the carbohydrates inulin, sucrose and the monosaccharides fructose and glucose, are subjected to hydrolysis and/or depolymerisation (inulin) or caramelisation (sucrose, monosaccharides) during the roasting process. Cyclisation and condensation reactions with nitrogenous components (proteins, amino acids) occur (Maillard reaction). Reactions with lipid degradation products are possible. Volatile and non-volatile products, water soluble and insoluble, are produced, for example furfural and 5-hydroxymethylfurfural (Pazola, 1987).
Glucose and fructose were found to be present at all roasting temperatures. Their content increased up to 160 °C due to degradation of inulin, but decreased at higher roasting temperatures. The content of oligosaccharides from inulin decreases steadily during roasting: from 35% (based on dry matter) in the starting material to 5% in chicory roasted at 190 °C. The optimum roasting temperature was 160-170 °C, when the reducing sugar content reached a maximum value (Pazola, 1987).

2.2.3 *Other compounds*

2.2.3.1 Nitrogenous compounds

Chemical processes, which affect proteins during roasting are: Maillard

reactions, i.e. carbonyl-amino reaction between amino acids, proteins and sugars, hydrolysis of proteins to peptides and amino acids, and pyrolytic decompositon of amino acids. Thus volatile compounds are generated, but also water-soluble and insoluble coloured products are formed from above reactions (Pazola, 1987). No free amino acids were found in chicory roots roasted at 180 °C or higher temperatures. Water is also a byproduct of the Maillard reaction.

2.2.3.2 Lipids

No literature was found on the lipid content of roasted chicory roots. Lipids undergo some degradation reactions during roasting, which affect the volatile composition and taste of the roots after thermal treatment.

2.2.3.3 Minerals

Ash content of roasted chicory root is affected by the roasting temperature: an increase is seen. Due to water loss, dry matter content increases during roasting; the increase in ash content can be explained by the increase in dry matter content.

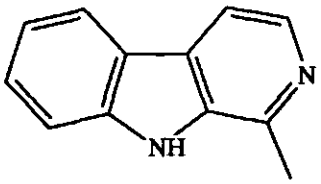
2.2.3.4 Organic acids

The amount of malic, citric, and tartaric acids decreases during roasting of chicory roots, and is dependent on the roasting temperature (Pazola, 1987; Gaber & Maier, 1989, 1990). Pazola (1987) found more acetic, formic, propionic, and lactic acids after roasting.

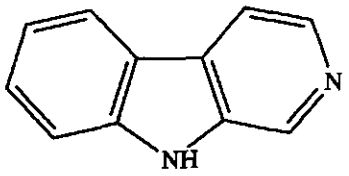
Gaber & Maier (1990) also determined the changes in percentage of the main acids of roasted chicory roots during wet storage, and during storage of chicory brews at elevated temperatures. In all solid samples and in all brews the titratable acidity and the contents of all organic acids measured, increased during storage.

2.2.3.5 Alkaloids

Proliac & Blanc (1976) isolated and identified from roasted chicory roots two beta-carbolines, namely harman and norharman (Fig. 2.9). They gave no evidence that these compounds contribute to the taste of roasted chicory.



harman



norharman

Fig. 2.9 Alkaloids isolated from roasted chicory roots.

2.2.3.6 Chlorogenic acids

Chlorogenic acids (CQAs) are partly degraded during roasting. In coffee beans 50-80% of the total CQAs may be destroyed during the roasting process (Macrae et al., 1987).

Haffke & Engelhardt (1986) determined chlorogenic acids in roasted chicory roots with HPLC. The concentrations ranged from 0.08 to 0.33 g/kg dry weight. In dried chicory a total amount of CQA of 1.93 g/kg was determined. This means a loss of CQAs during roasting of chicory roots between 83-96%.

Haffke & Engelhardt (1986) found one isomer in roasted chicory, namely 3-CQA, the other CQAs were below the detection limit. Clifford et al. (1987) found 5-CQA, and occasionally 4-CQA.

2.2.3.7 Coumarins

The effect of roasting on the coumarin content has not been studied, but it is likely that coumarins are not stable during the roasting process.

2.2.3.8 Volatile compounds

The first analysis of roasted chicory flavour compounds was published in 1930 by Reichstein & Beitter (Pazola, 1987). A distillate of fresh roasted chicory root was investigated, in which 15 compounds were detected. High amounts of furfural and 5-hydroxymethylfurfural (HMF), characteristic products of Maillard reactions, were found in the distillate.

Kawabata & Deki (1977) steam distilled roasted chicory roots and the composition of the distillate was detected by gas chromatography-mass

spectroscopy (GC-MS). Three pyrazines, 3 benzothiazoles, 6 aldehydes, 5 furans, 5 aromatic hydrocarbons, 3 phenols, 6 organic acids and 2 other components were identified. They stated that acetophenone was characteristic for roasted chicory root. In 1982 Sannai et al. identified 32 components in the extract of roasted chicory roots after steam distillation, 23 of which are reported as novel. They found a high amount of HMF and furfural in their chicory extract. In addition, palmitic acid, linoleic acid, and their methyl esters were found in large quantities in the roasted roots. However, no acetophenone was found in their distillate.

The flavour of a chicory brew is composed of volatile compounds and non-volatile taste components. Both are formed during the roasting process. A typical "spicy-peppery" flavour note has been described for chicory brews, but to date this does not appear to be related to any particular compound. With respect to the basic taste sensations, the acidity can be related to the organic acids present. The bitter taste of roasted roots is not so easily related to a specific compounds, since the bitter sesquiterpene lactones are assumed to be degraded during the roasting process (Pazola, 1987).

CHAPTER 3

BITTERNESS IN FOODS

3.1 Introduction

Bitterness is one of the four major taste sensations which are identified with the tongue and other parts of the mouth. The other sensations are saltiness, sourness, and sweetness. The mouth is also the major site of the responses to astringency, pungency, and "meatiness". The substances involved in all these sensations have a number of characteristics in common that distinguish them from substances commonly associated with odours. Taste substances are usually polar, water-soluble, and non-volatile. Besides their necessary volatility, odour substances are generally far less polar and elicit a much broader range of flavour sensations (Coultate, 1989).

Some naturally occurring bitter compounds known since antiquity have been used in certain healing herbal recipes and medicines. Therefore, bitterness is sometimes associated with medicines and pharmacological efficiency (Brieskorn, 1990).
By tasting bitter substances in foods and beverages, aversion arises because bitterness is perceived as a danger signal. Alkaloids present in many plants taste bitter and are toxic (Coultate, 1989).
However, it should be pointed out that all bitterness is not objectionable. In food products such as beverages (tonic water, beer or grapefruit juice), some bitterness is desirable. If these products exhibit no bitterness, they are usually judged as unacceptable. Furthermore bitterness at very low levels can impart a certain "cleanness", particularly to sweet flavours (Rouseff, 1990).
Bitter compounds may also be produced during processing of foods. For instance, amino acids and/ or peptides, which are formed during the proteolysis of milk proteins are primarily responsible for bitterness in for

example hypoallergenic infant formulas. Bitterness in cheese is an off-flavour and is thus regarded as a sensory defect. Maga (1990) reviewed both thermally promoted Maillard-type reactions and the interaction of enzymes in relation to bitterness in food as well as in model systems.

It has also been observed that the ability to detect bitterness varies widely within the population. Some individuals are extremely sensitive to bitterness, whereas others can detect bitter compounds only at very high concentrations. Since many naturally occurring toxins are bitter, one can speculate that the universal aversion to bitterness is the result of a primeval survival trait (Rouseff, 1990).

Bitterness of foods can be expressed by a wide range of molecules, with varying sizes and functional groups. Bitterness can be found in aliphatic or aromatic compounds, straight chained or polycyclic compounds, glycosides or aglycons with just about every functional group. Some inorganic salts and oxides, such as CaO, KCl, $MgSO_4$, have also a bitter taste.
In theory any type of molecule can exhibit bitterness, yet a slight structural modification of a bitter molecule can render it nonbitter. Therefore the ability to predict bitterness from molecular composition is rather limited.
Recently Belitz & Wieser (1985) reviewed the most important types of bitter compounds of plant origin. Among the naturally occurring bitter principles, these are by far the larger group.

3.2 Taste receptors

Human taste sensing organs are found on the surface of the tongue. Bitterness receptors are particularly concentrated on the back of it. Taste sensing organs are also found in the mucosa of the cheeks (primarily in young children), in the anterior part of the soft palate, in the retropharyngeal wall, in the inner surface of the epiglottis and on the tip of the larynx (Brieskorn, 1990).
The sense organs of the tongue can be visualized as different types of little hills called papillae. The papillae contain clusters of taste buds. The taste

buds cannot further be subdivided into different types. They are constantly depleted and renewed. The avarage lifetime of a taste cell is approximately 250 hours.

The sensation of bitterness requires a reaction between the bitter compound (stimulant) and the taste cell. Within the taste cell receptors can be characterized. A receptor has functional groups and a certain specified conformation to bind the active (bitter) compound. If the conformation of a compound which comes in contact with the cell, is sufficiently specific and has sufficient binding energy, then the microstructural conformation of the receptor membrane undergoes a rapid, short term (1 msec) change. This is converted into an electric signal, because the potential of the cell membrane is changed by the interaction between stimulant and receptor cell.

Taste thresholds for most bitter compounds show a Gaussian distribution, but those for phenylthiocarbamide (PCT), containing the N-C=S group, are bimodal. Thus people can be grouped into either taster or nontaster population according to their ability to recognize PCT as bitter. This strongly suggests that there are at least two different types of bitterness receptors (Gardner, 1979). Probably PCT tasters have both types of receptors, whilst one of the receptors is absent with non PCT tasters.
The same sort of model accounts for the observations that the population in general can also be divided into bitter sensitive and insensitive tasters, where the insensitives could have fewer of the receptors near the surface and hence require higher concentrations of the tastant to ensure penetration into the deeper-lying receptor (Gardner, 1979).

3.3 Theories on bitterness

Bitterness can be expressed by a wide range of molecules with varying sizes and functional groups. There is a relationship between the bitterness and structure of selected molecular families. However, they are generalizations:
a. Molecular size. Sodium chloride has a salty taste, whereas sodium iodide is bitter;
b. Functional group. Electron-withdrawing groups such as nitro group may

cause bitterness;
c. Sugar position. Naringin and neohesperidin are both citrus flavanone glycosides. The bitterness of these compounds depends on the amount of sugar units (two or one) in the carbohydrate part of the molecule, and on the place of the glycoside bond between the two sugar residues;
d. Decrease of hydrophilic character of the molecule;
e. Stereochemistry. L-Amino acids taste bitter whereas their corresponding D-enantiomers are sweet (Brieskorn, 1990).

Hydrophobicity of molecules correlates with intensity of bitter taste (Belitz & Wieser, 1985). Surface tension seems to be inversely related with the bitter taste of aqueous solutions. Also Gardner (1979) related lipophilicity of a molecule, the lipid solubility of the molecule as a whole, with their relative bitterness.

Some workers have tried to give a more fundamental basis to relate bitterness with chemical structure. Especially amino acids and sugars have been investigated, because these compounds possess sweet as well as bitter taste.

3.3.1 *Q-value*

In 1962 Tanford proposed a model for calculation of the difference in free energy (δF) between the native and unfolded forms of a protein molecule in solution. The major term in the expression for δF arises from the increase in entropy which accompanies unfolding; then δF is negative. In water where a compact globular conformation is stable, intramolecular interactions exist which give a positive contribution to δF. When amino acids with non-polar side chains are dissolved in water, the same interactions occur. The magnitude of these interactions can then be estimated from relative solubilities of appropriate amino acids in water and other solvents, such as ethanol (Tanford, 1962). It is a measure for hydrophobicity. The δf value of an amino acid (in cal/mol) can be calculated from these solubility data. The factor δF of a peptide is calculated from the sum of the contributions of the single amino acid residues:

$$\delta F = \Sigma \delta f$$

Ney (1971) adapted this value to study bitter peptides. He defined a value Q, the average hydrophobicity of the peptide:

$$Q = \frac{\Sigma \delta f}{n}$$

in which n is the number of amino acid residues in the peptide, and δf represents the contribution of a single amino acid side chain to the hydrophobic interaction of the protein (Ney, 1971; 1979). Peptides with Q-value lower then 1300 were found non-bitter, whereas bitter peptides had a Q-value greater then 1400. However, this principle is valid for peptides with molecular weights up to approximately 6000 Daltons; above this limit peptides with a Q-value above 1400 are also not bitter (Ney, 1979).

Wieser & Belitz (1975) investigated the bitter taste of various amino acids, amino acid esters, and N-acyl amino acids. They found for amino acids and derivatives that the amino group is essential for the bitter taste, which interacts with the nucleophilic groups of the receptor cells. The influence of the carboxylic group was negligible. Not only hydrophobicity of the molecule is important for the bitter taste, but also steric factors, which influences the interactions with the receptor cells.

The same model was proposed for peptides (Wieser & Belitz, 1976). The amino group acts as polar group which interacts with the receptor cell. Hydrophobicity of the peptide, and the number and nature of the side chains of the molecule are related with bitterness intensity (Wieser & Belitz, 1976).

They postulated that one polar (electrophilic) group and one hydrophobic group are essential requirements for the bitter taste of amino acids, amino acid derivatives, and peptides (Wieser & Belitz, 1975, 1976). For amino acids both groups must be arranged in a defined manner. The model corresponds to the bitterness of all hydrophobic peptides, and is independent on the amino acid sequence and configuration.

Wieser & Belitz (1976) calculated the hydrophobicity of the side chain of an amino acid as the difference between the free energy of the amino acid and

that of glycin. The mean hydrophobicity of a peptide was then calculated from the sum of the hydrophobicities of the amino acids of the peptide and the hydrophobicity of the corresponding peptide consisting of only glycine:

$$\delta F_{peptide} = \delta F_{(glycin)n} - \Sigma \delta f_{amino\ acid}$$

which gives according to Wieser & Belitz (1976) a more accurate value for hydrophobicity of a peptide and an estimate for bitterness.

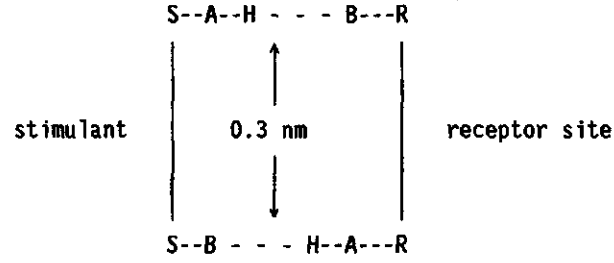
Adler-Nissen (1988) studied bitterness intensity of protein hydrolysates as function of degree of hydrolysis, and demonstrated that Ney's Q-rule is theoretically unfounded.
He found that hydrophobicity of bitter peptides is not a simple function of the average hydrophobicity of the amino acids. The bitterness level of protein hydrolysates could be predicted and calculated from the molar concentration and the average hydrophobicity of the hydrophobic, i.e. 2-butanol extractable, peptides. The degree of hydrolysis, defined as the percentage of peptide bond cleaved, is the third variable which influences bitterness (Adler-Nissen, 1988).
A hydrophobic amino acid gives rise to the strongest bitterness when it is positioned in the interior of the peptide, depending on chain length. For instance, hydrophobic side chains of a protein (polypeptide) are physically hindered in interacting with the taste receptors and thus cannot cause bitterness. A slightly weaker bitterness is observed when the hydrophobic amino acid is situated in the terminal position, and the lowest bitterness intensity is found as free amino acid. Thus, a dipeptide composed of two hydrophobic amino acids will taste much more intense than an equimolar solution of the same amino acids (Adler-Nissen, 1988).

3.3.2 *AH-B theory*

Shallenberger and co-workers studied the relationship between carbohydrate structure and sweet taste. They assumed that two adjacent hydroxyl groups in the sugar molecule are responsible for the sweet taste, and that the taste intensity is inversely related to the extent of hydrogen bonding between

these groups and, consequently that it depends upon their mutual conformation.

In 1967 Shallenberger & Acree postulated a molecular theory of sweet taste, in which all sweet agents (stimulants) possess a system of the type AH-B (proton-donor respectively proton-acceptor) in their structures, wherein A and B are electronegative atoms separated by a distance of 0.24-0.4 nm (Scheme 3.1).



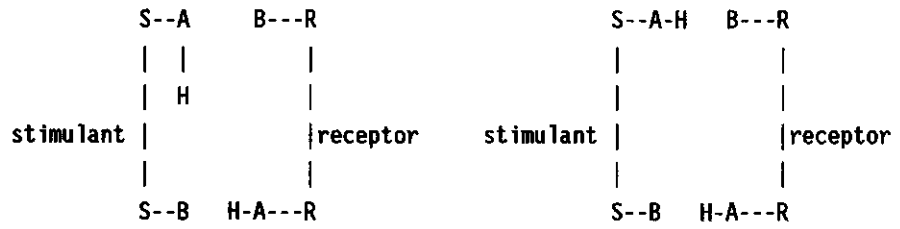
Scheme 3.1 AH-B model for sweet taste according to Shallenberger & Acree (1967).

The theory further assumes that receptor sites, which are involved in the interactions contributing to the generation of sweet taste, also possess a complementary AH-B system, and thus that the interaction between the two systems is based upon the simultaneous formation of two inter-system hydrogen bonds (Beets, 1978).

In the AH and B system two different molecules may contribute to the sweet taste by one acting as proton-donor to the receptor site and the other as proton-acceptor (Belitz & Wieser, 1985).

Kubota & Kubo (1969) postulated a similar AH-B system involved in the generation of bitterness (Scheme 3.2). They examined the taste and structure of a large number of bitter and tasteless diterpenes from *Isodon* species. In each bitter compound they investigated, proton donor and proton acceptor moieties were present, however the intramolecular distance between one AH-B pair is 0.15 nm. Also proton donors and proton acceptors of the taste bud can

act as receptor site, and these probably form hydrogen bonds with the bitter unit. In case of bitterness, however, the proton donor at the receptor site must be strong enough to sever the hydrogen bonding in the substrate site (Kubota & Kubo, 1969). For the bitterness of diterpenes from *Isodon* an intramolecular hydrogen bond was a requisite.



Scheme 3.2 AH-B model for bitter taste according to Kubota & Kubo (1969).

In the AH-B system two polar groups with definite steric arrangement are postulated as essential groups for sweet-tasting molecules, which may be supplemented by a hydrophobic group for intensifying the sweet taste. In contrast, bitter compounds need only one polar group (electrophilic or nucleophilic), and a hydrophobic group. The taste threshold of bitter compounds depends mainly on the apolar moiety, but is also related to steric factors and to the charge distribution within the molecule (Belitz et al., 1988).

The proposed proton-donor proton-acceptor system (AH-B) by Shallenberger & Acree (1967) gives evidence to the hypothesis that sweet and bitter receptors are strongly related. Based on investigations of aspartame analogues it is assumed that the bitter and sweet qualities are recognized by the same taste receptor and that the receptor site easily discriminates between bitter and sweet taste by the different combinations between AH-B (Belitz & Wieser, 1985).

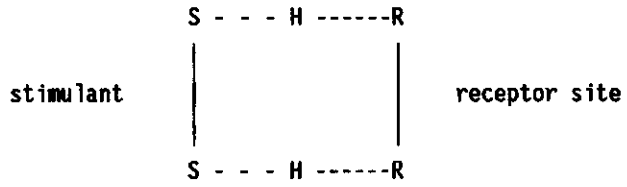
However, this model is not fully supported by studies on bitterness in other compounds (Gardner, 1979). He postulated that lipophilicity is an important parameter in inducing a bitter taste. For example intramolecular hydrogen bonds correlate with bitterness because they increase the relative

lipophilicity of a molecule. Fewer groups are then available to form intermolecular hydrogen bonds to the aqueous medium of saliva. Hence the degree to which the molecule will penetrate into the cell membrane will increase, giving it a better chance of reaching the bitterness receptor.

3.4 Bitterness of sesquiterpene lactones

The bitter taste of sesquiterpene lactones present in chicory roots, lactucin, lactucopicrin, 8-deoxylactucin, and their dihydro derivatives can in part be explained by above mentioned theories.
The bitter components are usually extracted from the roots with an apolar solvent. They are slightly soluble in water except Lp. This compound can be purified by recrystallisation in water (Holzer & Zinke, 1953).
Lp and its dihydro analogue are the largest molecules of the sesquiterpene lactones investigated. Both molecules elute with RP-HPLC after Lc and 8dLc. With this chromatographic method only hydrophobic interactions of the solutes with the stationary phase are possible (Fallon et al., 1987).
Lc has at C-8 a hydroxyl group and 8dLc a hydrogen atom (see Fig. 2.1). Lp has the largest substituent at C-8, a p-hydroxyphenyl acetic acid group. Therefore Lc is the most polar and should have the lowest bitter intensity, and Lp the highest. Similar bitterness scores are expected for the dihydro analogues.

Intramolecular AH-B interactions are theoretically possible in the sesquiterpene lactones investigated between the O-atom of the lactone ring and the -OH group at C-14, and between the carboxylic group at C-2 and the methyl group at C-15. These interactions are similar for the three components, and should not give any difference in bitter intensity.
According to Beets (1978) a single lactone group in a sesquiterpene molecule seems to suffice for bitter taste. He proposes a modified AH-B theory or interaction complex, in which the donor functions are entirely supplied by the accommodating receptor site (Scheme 3.3).
The lactone group is a possible partner in above interaction complex. He states that bitterness must be due to the presence of more or less polar moieties in the structure, possible in combination with lipophilic centres



Scheme 3.3 AH-B model for bitter taste as proposed by Beets (1978).

(Beets, 1978).
Also the lactone group is identical for the three sesquiterpene lactones, and should therefore not contribute to different bitterness intensities of the compounds.

The conclusion is that the bitter taste of sesquiterpene lactones from chicory root, especially lactucin, lactucopicrin, and 8-deoxylactucin, can be explained by their hydrophobic character, and partly by the AH-B theory. Difference in bitter intensity will be caused by the difference of the substitutes at the C-8 atom.

CHAPTER 4

EXTRACTION OF SESQUITERPENE LACTONES

4.1 Introduction

Various extraction solvents have been used for the isolation of bitter compounds from lactucarium obtained from *Lactuca virosa* as well as other *Lactuca* sp., and from chicory roots. There are many publications in this area.

Späth et al. (1939) isolated lactucin (Lc) from dried lactucarium by soaking the powder in water, adjusting to pH 1, and partitioning into diethyl ether. Others used this method too for the isolation and purification of lactucin (Barton & Narayanan, 1958; Schenck et al., 1964).

Schenck & Wendt (1953) isolated lactucopicrin (Lp) from lactucarium by Soxhlet extraction using chloroform (30 h). Schmidt (1940) used methanol to isolate lactucopicrin and lactucin from fresh and dried milky juice of *Lactuca virosa* and for the isolation of these sesquiterpene lactones from other Compositae, including *Cichorium intybus*.

Schenck et al. (1961) isolated both lactucin and lactucopicrin from whole *Lactuca virosa* plants with 50% methanol acidified with 38% HCl (1 ml per 100 ml of 50% MeOH). They claimed that extraction at low pH (pH 2) prevented the oxidation of Lp by endogenous *Lactuca* enzymes, and that consequently at this pH the highest yield of Lp was obtained. Pyrek (1977) extracted *Lactuca serriola* roots with methanol and isolated Lc, Lp, 8-deoxylactucin (8dLc) as well as 11(S),13-dihydro-8-deoxylactucin (dH8dLc). Mahmoud et al. (1986) extracted the aerial parts of *L. sativa* with Et_2O-petrol (1:2) and isolated Lc, Lp and 11(S),13-dihydrolactucin (dHLc). Lactucopicrin, dHLc, and lactucopicriside (glycoside of lactucopicrin) were extracted from *Lactuca laciniata* roots with water (Nishimura et al., 1986).

Holzer & Zinke (1953) and Zinke & Holzer (1953) extracted lactucin and lactucopicrin with ethanol from the milky juice of fresh chicory roots, which

was stabilized with methanol. Dolejs et al. (1958) and Rees & Harborne (1985) used this method for isolation of lactucin, lactucopicrin as well as 8-deoxylactucin from chicory.
Pyrek (1985) used fresh chicory roots, extracting them with methanol or acetone. El-Masry et al. (1984) isolated from the roots of *Cichorium pumilum* (a subspecies of *C. endivia*) two new sesquiterpene lactones of the guaianolide type, 10(S)-hydroxycichopumilide (Fig. 2.4) and its 11(S),13-dihydro derivative (ratio ca. 5:3) with a mixture of diethyl ether-petrol.
Seto et al. (1988) isolated several sesquiterpene lactones, including lactucin and lactucopicrin from fresh *Cichorium endivia* and *C. intybus* roots, using methanol under reflux.
The influence of different forcing methods on the bitter constituents of chicory was studied using freeze dried roots and heads, by extraction with 50% methanol acidified with HCl. No concentration of HCl was given (Dolezal, 1976). Chicory heads proved to have a maximum of bitter components after forcing of the roots in synthetic foam flakes without cover. In the chicory roots an increase of bitter compounds was found during forcing.
Although various extraction procedures are described, only Pyrek (1985) compared an acetone extraction with a methanol extraction of chicory roots. The composition of both extracts was similar, however the methyl ester of lactucopicrin was only present in the MeOH extract. The other sesquiterpene lactones did not give analogue compounds during methanol extraction. Lactucopicrin therefore is particularly susceptible towards lactone ring opening in methanol.

We wanted to isolate the sesquiterpene lactones from chicory roots. HPLC methods were first developed to analyse, separate and identify the isolated compounds.
Sesquiterpene lactones isolated from chicory have a rather apolar character. High extraction yields are therefore expected when the roots are extracted with relatively apolar solvents. However, literature data are not clear: polar and more apolar solvents have been used. No yields were given and no comparison between different extraction solvents were made.
In this study various extraction solvents were evaluated for their ability to isolate sesquiterpene lactones from chicory roots. The effect of storage conditions of the chicory roots was also investigated. Special attention was

given to storage at various temperatures, and the effect of drying and milling on the extraction efficiency.

4.2 Results and discussion

4.2.1 *Analysis*

Initially a rapid and simple procedure for analysing sesquiterpene lactones from chicory roots with reversed phase HPLC was developed with a water-methanol mixture as eluent (isocratic method). Three major peaks were characterized with MS and NMR: lactucin, 8-deoxylactucin, and lactucopicrin (Fig. 4.1 A). The spectra were identical with those of Pyrek (1977).
However, at least one of the peaks consisted of two components as distinguished by MS: 8dLc and a compound with molecular formula $C_{15}H_{20}O_3$ (mass spectrum *m/e* (70 eV) 248 (M^+), 233, 230, 204, 191, 175, 163 and 141). This mass spectrum was compared with that of cichoriolide A (Fig. 2.2) described by Seto et al. (1988). It seems not unlikely that the two compounds are identical. This compound was not further identified.
It was also not possible to explain the increase of Lc and 8dLc during enzymatic liquefaction of chicory roots (see Chapter 5). Therefore a gradient HPLC method (water-methanol gradient) was developed for accurate determination and identification of the compounds present in chicory roots. Fig. 4.1 shows HPLC chromatograms of chicory root extract obtained by the first method (Fig. 4.1 A) and by gradient elution (Fig 4.1 B), the latter consisting of a large number of components many of which have not been identified. Two of the components were investigated further: one before dHLc (peak Q) and one before 8dLc (peak T) (see Chapter 5).
With the second developed HPLC method (gradient elution) the three major peaks of Lc, 8dLc and Lp obtained by the first method could be separated in two new ones: the already mentioned compounds and their dihydro derivatives; 11(S), 13-dihydrolactucopicrin (dHLp) was identified and described for the first time. The MS and NMR spectra of dHLc and dH8dLc were identical to those of Seto et al. (1988) and Pyrek (1977). The spectral data for dHLp are described in Chapter 4.4.3.
The reproducibility of both HPLC methods for the sesquiterpene lactones

analysed was satisfactory: the coefficient of variation of both methods was between 2 and 5% for all sesquiterpene lactones investigated (Leclercq, 1984; van Leeuwen, 1989; number of replicates of one run was n=11 and n=7 respectively).

4.2.2 *Extraction solvents*

The extraction of the bitter constituents of the roots was first carried out according to Späth et al. (1939) with diethyl ether for 30 h, combining a solid-liquid (roots-water) and liquid-liquid (water-diethyl ether)

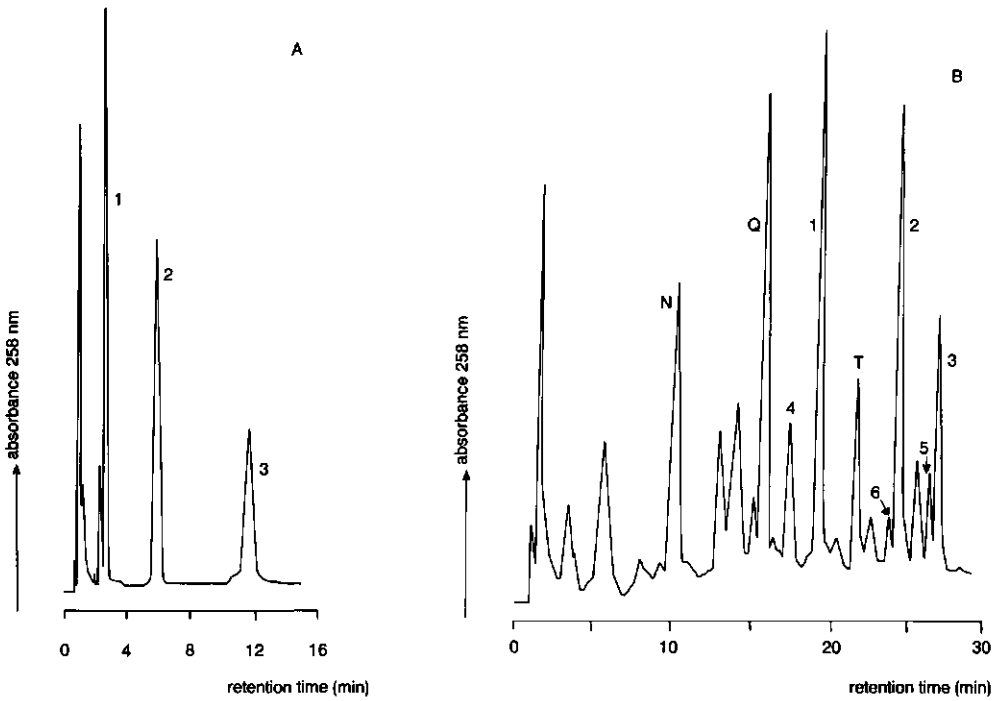


Fig. 4.1 Reversed phase HPLC (A) and gradient HPLC (B) chromatogram of chicory root extract.
1=Lc; 2=8dLc; ; 3=Lp; 4=dHLc; 5=dHLp; 6=dH8dLc Q=peak Q; T=peak T; N=peak N.

extraction. However, this extraction procedure has a number of disadvantages:

1. The extraction with diethyl ether is time consuming, thus the method is not suitable for rapid quantification of sesquiterpene lactones in chicory roots or the residues formed after enzymatic liquefaction.
2. Relatively large samples of roots or residues are needed with the apparatus used (100-200 g).
3. Further extraction of the chicory roots after 30 h with a fresh portion of diethyl ether using the method developed by Späth et al. (1939) enabled the isolation of more sesquiterpene lactones, so the extraction was not exhaustive.
4. The amount of sesquiterpene lactones extracted from the roots with the above mentioned method did not correspond with the amount of these compounds obtained after enzymatic liquefaction: more lactucin and 8-deoxylactucin was found after liquefaction than in the untreated raw material. This phenomenon was the reason for improving the extraction procedure of sesquiterpene lactones from chicory roots (as described further in this Section), for improving the method of analysis (see Section 4.2.1), and for concentrating on the precursors of Lc and 8dLc and the enzyme system of chicory roots (Chapter 5, 6).

The extraction solvents water, MeOH, EtOH, chloroform, and acetone, were evaluated for their ability to isolate sesquiterpene lactones from chicory roots. All extracts, except the chloroform extract, consisted of mainly polar compounds eluting before Lc in the HPLC chromatogram. The amount of Lc was less compared with an diethyl ether extract prepared according to Späth et al. (1939). No 8dLc and Lp could be detected (Netjes, 1985). Satisfactory results were obtained with repeated extraction with chloroform, however, lactucin was not completely extracted with this solvent from the roots nor from the residues of the roots after enzymatic liquefaction.
In a subsequent experiment four different solvents for extraction and isolation of the bitter compounds of fresh chicory roots were tried and compared qualitatively with TLC: ethyl acetate, ethyl acetate with 10% isopropanol, butanol, and chloroform. Ethyl acetate gave the highest yield of the three sesquiterpene lactones investigated. Analysis with gradient HPLC showed that this extract contained also the 11(S),13-dihydro analogues of the

sesquiterpene lactones investigated.

The effect of polarity of the extraction solvents on the quantitative recovery of sesquiterpene lactones from chicory roots was measured with gradient HPLC. Extraction solvents used were ethyl acetate (with and without addition to the roots of 2% Na_2SO_4), acidic ethyl acetate, methyl acetate, and a MeOH/H_2O (55-45 or 80-20) mixture.

Addition of salt (Na_2SO_4) during the ethyl acetate extraction decreases the yield of lactucin and its 11(S),13-dihydro analogue. The salt was added to the fresh roots prior to extraction, thus probably lowering the activity of native enzymes of the roots, to give a lower yield of these compounds.

In the acid ethyl acetate soluble fraction more 11(S),13-dihydrolactucin was found compared to the ethyl acetate alone, and peak Q decreased. Peak Q, eluting before dHLc, is one of the components which was investigated further (Chapter 5).

Methyl acetate as solvent gave a similar extract as EtOAc, however, all peaks were smaller.

The extractions with MeOH-H_2O mixtures were less selective and gave extra peaks compared with the ethyl acetate extraction, especially polar compounds eluting at the beginning of the HPLC chromatogram.

Ethyl acetate appeared to be the best extraction solvent for the isolation of sesquiterpene lactones from chicory roots. The reproducibility of the extraction of these compounds from chicory roots with EtOAc was established by four replicate analyses of the same sample. For Lc, dHLc, 8dLc and dH8dLC a satisfactory precision was obtained after combining two successive extracts (coefficient of variation between 9 and 21%). However, the coefficient of variation increased with apolarity of the compounds, which might be due to the decreasing solubility of the apolar compounds in the MeOH/H_2O solvent in which the EtOAc solvent solubles were dissolved. The peak height of apolar compounds eluting in the end of the chromatogram, for example lactucopicrin, is relative small compared to more polar components measured, and more tailing occurs, both increasing the coefficient of variation. This decrease in precision of especially apolar compounds is also seen for other extraction solvents.

4.2.3 *Effect of processing of raw material*

Until now there are no publications on the effect of processing (i.e. freezing, milling degree, drying) on the extraction of sesquiterpene lactones from chicory roots. Therefore the effect of storage, milling, and drying on the extraction yield was investigated.
First storage conditions of the roots were investigated. The extraction of fresh chicory roots was compared to chicory roots which were extracted after

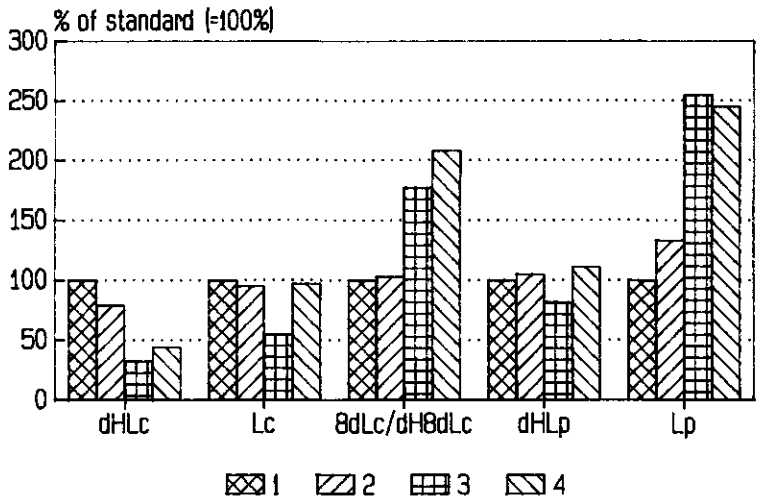


Fig. 4.2 Effect of storage conditions and milling on extraction efficiency of chicory roots.

1 *Standard extraction procedure, no storage*
2 *Extraction after an extra milling step with sand*
3 *Extraction after 1 month storage at -30 °C*
4 *Extraction after 1 month storage at 1 °C*

storage at 1 °C for one month, and chicory roots which were stored frozen for one month (-30 °C).
The amount of extracted sesquiterpene lactones is influenced by storage (1 °C or frozen): an increase is seen for 8-deoxylactucin, its 11(S),13-dihydro derivative, and lactucopicrin, while the amount of 11(S),13-dihydrolactucin decreases (Fig. 4.2).
During storage of the chicory roots at low temperature native enzymes could possibly convert polar compounds, e.g. glycosides of the sesquiterpene lactones investigated, into less polar aglycons, which elute later in the chromatogram. Freezing breaks the cell membranes of the chicory roots, thus increasing the extraction efficiency.

In a second experiment chicory roots were first milled with sand before extraction with EtOAc. The additional milling of the roots decreased the amount of dHLc, but increased that of Lp (Fig. 4.2), however, the latter amount falls within the error of analysis.
Milling with sand increases the total surface of the roots exposed to the solvent and thus the extraction might be more efficient. However, the total dry matter of material to be extracted increases substantially when sand is added. Therefore no increase in extraction efficiency is seen (see next part of this section). Thus a similar extraction efficiency with regard to isolation of sesquiterpene lactones is obtained compared to the standard extraction procedure.

An EtOAc extract of air dried chicory roots (70 °C, 24 h) contained almost no sesquiterpene lactones. The procedure decreased tremendously the compounds of interest. After roasting of chicory roots (1 h at 130 °C), no bitter sesquiterpene lactones could be detected: presumably they were degraded (Stoltze, 1987; Pazola, 1987).
After steam blanching of the chicory roots (for 1 min) no sesquiterpene lactones could be extracted with a chloroform extraction.
A low extraction yield was also obtained from freeze dried chicory roots extracted with ethyl acetate: almost all components decreased by about 50% compared to fresh roots (Fig. 4.3).
The use of freeze dried chicory samples is not unusual: Dolezal (1976) used freeze dried samples of chicory roots and heads and extracted them with 50%

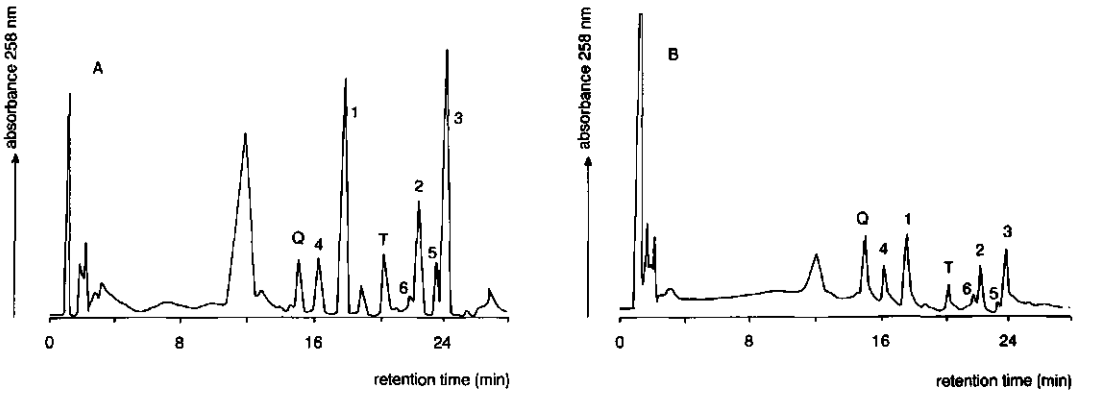


Fig. 4.3 HPLC chromatograms of EtOAc extracts from fresh chicory root (A) and freeze dried chicory root powder (B).
1=Lc; 2=8dLc; 3=Lp; 4=dHLc; 5=dHLp; 6=dH8dLc; Q=peak Q; T=peak T.

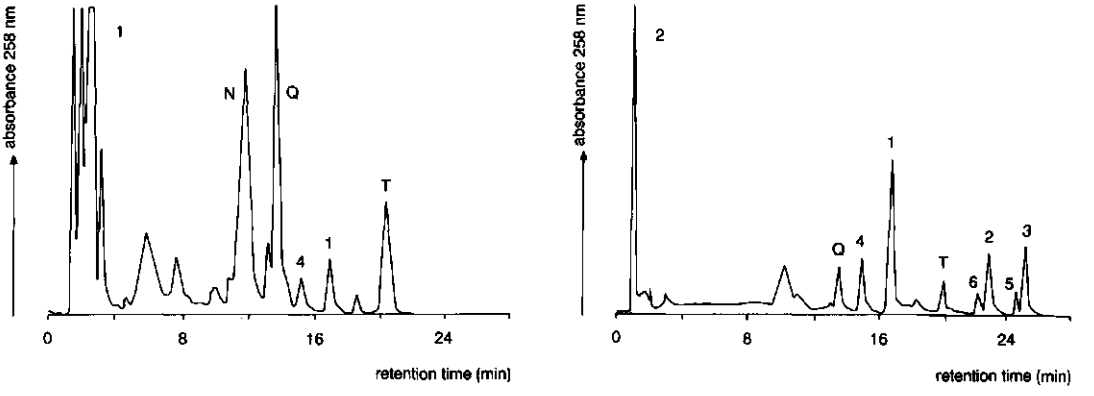


Fig. 4.4 HPLC chromatograms of EtOAc extract from freeze dried chicory root powder after soaking with water.
1=H_2O layer; 2=EtOAc layer.
1=Lc; 2=8dLc; 3=Lp; 4=dHLc; 5=dHLp; 6=dH8dLc; Q=peak Q; T=peak T; N=peak N.

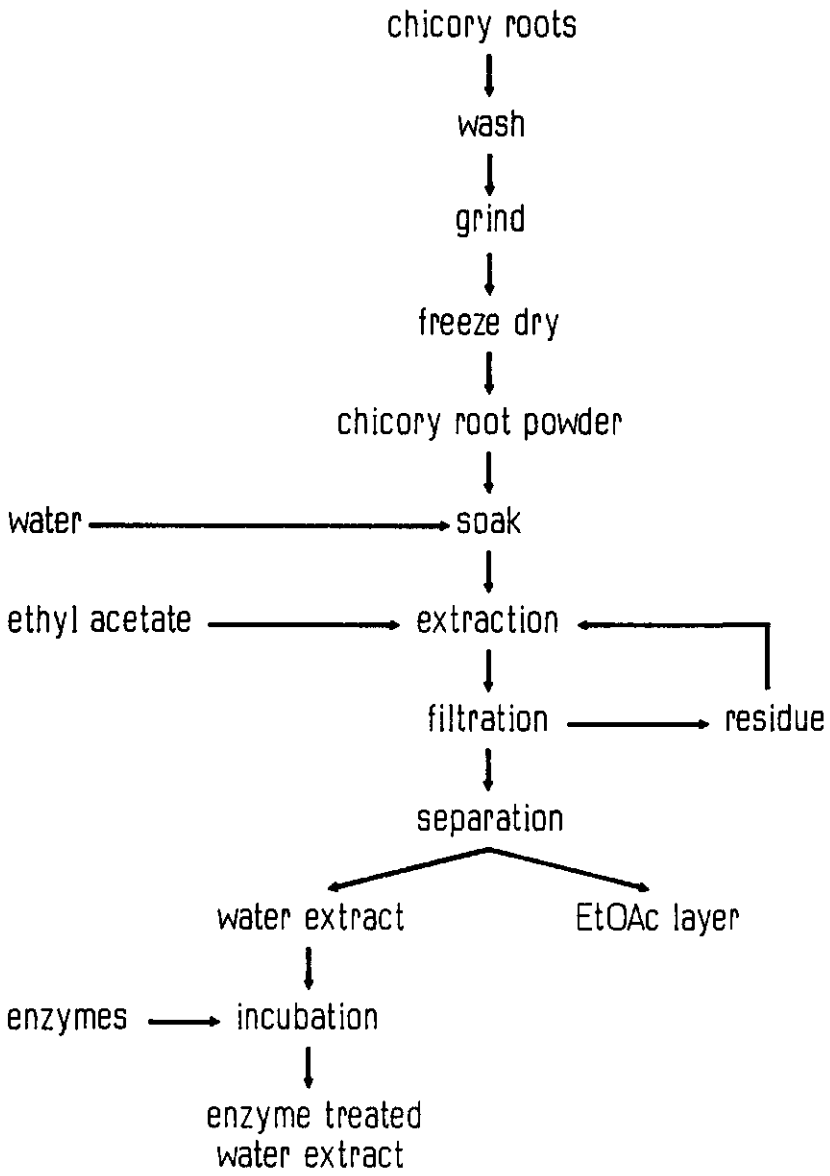


Fig. 4.5 Flow diagram for extraction of sesquiterpene lactones from chicory roots via a freeze drying step.

methanol acidified with HCl at 70 °C. No problems were mentioned with this extraction, probably because a sufficient amount of water was available.

Therefore freeze dried chicory root powder was first soaked with water (15 min) before extraction with ethyl acetate (see Fig. 4.5 for flow diagram). The yield of sesquiterpene lactones increased. The amount of water added to the dried chicory root powder was also found to be important: higher yields were obtained with twice the natural quantity of water added to the dried powder. The obtained water layer consisted mainly of (unknown) polar compounds, probably precursors of bitter sesquiterpene lactones (Fig. 4.4). This increase of total yield of sesquiterpene lactones could be due to a moisturizing effect of the dried roots, by which the extraction with ethyl acetate is improved. Carbohydrates are the main components of chicory roots. They do not dissolve in EtOAc, so that they prevent the sesquiterpene lactones to come into contact with the solvent. In an aqueous solution more Lc, 8dLc and Lp could be detected in the presence of polysaccharides (Leclercq et al., 1988). Späth et al. (1939) stated that soaking of lactucarium with water gives a considerable amount of sesquiterpene lactones in the water phase before extraction with diethyl ether, and thus suggests that soaking with water gives a higher yield of bitter compounds and/ or an increase in extraction efficiency. Water appeared to be necessary for extraction of the sesquiterpene lactones present in the roots.
Native enzymes can be activated during soaking of the dried roots with water. Sugars present in the roots will protect enzymes against activity losses, which is described for other, pure enzymes (Carpenter & Crowe, 1988). The effect of native enzymes on yield of sesquiterpene lactones is discussed in more detail in Chapter 5 and 6.

4.3 Conclusions

Ethyl acetate is the best extraction solvent of those tested for the isolation of sesquiterpene lactones from chicory roots, giving a simple procedure and giving reproducible extracts. However, the precision of the analysis of the apolar compounds (such as lactucopicrin) is less than that of the more polar compounds (e.g. lactucin).

Chicory roots extracts obtained from polar solvents, such as water, methanol, and acetone, consist of more polar components than ethyl acetate or diethyl ether extracts. Less Lc, 8dLc and Lp are found. Seto et al. (1988) used MeOH for chicory root extraction and found many (more polar) glycosides in the solvent solubles. It might be expected that glycosides are present in above described "polar" extracts, and that they elute at the beginning of the HPLC chromatogram.
Depending on which compounds have to be extracted from chicory roots, a suitable extraction solvent can be chosen. For polar compounds methanol/water mixtures are appropriate, for apolar compounds ethyl acetate or diethyl ether. The extraction with diethyl ether as solvent is not useful when a rapid assay of a sample is necessary, and is not exhaustive. Therefore the use of ethyl acetate is preferable.

Gradient elution with reversed phase HPLC is the most suitable method for determination and identification of sesquiterpene lactones from chicory roots (Fig. 4.1B). The first developed, isocratic method for rapid determination of bitter compounds (Fig. 4.1A) appears to be useful for screening of a chicory extract, for presence and elucidation of compounds, and might also be appropriate to associate sensory analysis of bitter sesquiterpene lactones with HPLC analysis. However, the three major peaks obtained with this rapid method consisted of at least two (and probably more) components.

Storage conditions, freezing, thawing, and the degree of milling, all influenced the total yield of sesquiterpene lactones investigated, and thus partly effected the reproducibility of chicory extraction. Especially the milling step is crucial for obtaining reproducible extracts. To inhibit enzyme activity it is recommended to grind the chicory roots at the lowest possible temperature, and to do it in the shortest time possible. Cryomilling seems to be the best option. A heat treament, for instance blanching, or drying at high temperature, (partly) degrades the compounds of interest.

4.4 Experimental section

4.4.1 *Raw material*

Fresh chicory roots (*Cichorium intybus* L.) were obtained from a grower in Veenendaal, the Netherlands, and stored at 1 °C before use. The roots were divided in several portions at random.

i. After arrival and milling fresh roots were immediately extracted, and the extracts obtained were compared with those from roots, which were stored at 1 °C or frozen for one month before use.

ii. Part of the chicory roots were milled, and the pieces obtained were frozen (-30 °C), freeze dried or dried in a hot-air oven with forced ventilation for 24 h at 70 °C.

iii. Chicory roots were cut into small pieces, and further reduced in size by milling with sand.

Chicory roots used for the extraction with diethyl ether, water, MeOH, EtOH, acetone and chloroform were cut into small pieces (about 4 mm^3) under liquid nitrogen and stored in portions of 100 g each at -60 °C.

Before use all chicory roots were cleaned to remove adhering soil.

4.4.2 *Extraction procedures*

A portion of milled and frozen chicory roots (200 g) was suspended in 600 ml of water at room temperature, and sodium chloride (20 g) was added. After 30 min, the suspension was extracted continuously for 30 h with diethyl ether in a liquid-liquid extractor according to Späth et al. (1939). After extraction, the diethyl ether was removed by evaporation and the residue dissolved in 50 ml of 95% ethanol.

Extraction of the chicory roots with methanol, ethanol, acetone or water was carried out as follows. Frozen chicory root pieces (10 g) were successively extracted for 5 x 4 h with one of the above mentioned solvents (20-25 ml) at room temperature. After each period of time the solvent was removed by filtration and replaced by a fresh portion. The five extracts were combined and the solvent was removed by evaporation. The residue of the extract was

dissolved in 1 ml of MeOH. This extraction was also carried out at the boiling temperature of the respective solvent. The eight thus obtained extracts were analysed for the sesquiterpene lactones (first method).

Extraction of chicory roots (10 g) with chloroform (20 ml) was carried out at room temperature. The material was extracted four times for 16, 4, 4, and 16 h respectively by shaking continuously. After each period of time chloroform was removed by filtration and replaced by a fresh portion. The four extracts were combined and the chloroform was removed by evaporation, after which the residue of the extracts was redissolved in 1 ml of MeOH.

Ethyl acetate extraction was carried out by boiling chicory roots (1 g) twice with a fresh portion of solvent (10 ml) for 15 min. The two extracts were combined and evaporated in vacuo. The residue obtained was redissolved in 10 ml of MeOH/H_2O (50-50).

Extraction with a mixture of methanol-water, methyl acetate, and acidified ethyl acetate, was performed as described for ethyl acetate. Acidified EtOAc was a mixture of 0.1 N HCl and EtOAc (10-90).
Unless stated otherwise ethyl acetate was used as the extraction solvent.
Extraction of freeze dried chicory root powder is described in Section 5.3.2.
All solvents were obtained from Merck, Darmstadt, Germany.

4.4.3 *Analysis*

Extracts obtained by diethyl ether, acetone, water, ethanol or chloroform extraction were analysed by isocratic, reversed phase HPLC (RP-HPLC) using a 10 cm x 8 mm Radial PAK C18 cartridge (Waters Assoc., no. 84720) with a C18 Guard-PAK pre column (no. 85824), which were pressurized in a radial compression Z-Module before use. The solvent was water-methanol (50-50) and the flow-rate was 2.0 ml/min. UV detection at 258 nm was used.
The extracts obtained by using EtOAc, acidified EtOAc, MeOAc, and MeOH-H_2O as solvent, were analysed by gradient HPLC using a Spherisorb C-18 column (250 x 4.6 mm, particle size 10 μm). Eluent A was methanol-water (5-95); eluent B was methanol-water (70-30). During one run of 25 min 100% eluent A decreased

linearly to 0%, and eluent B increased from 0% to 100%. The flow rate was 1 ml/min, and UV detection at 258 nm was used.

Deionized, double distilled water was used. The other solvents were of HPLC grade.

Ready-made silica gel TLC plates (60 F-254, Merck, no. 5719) were used with EtOAc as solvent. Detection was carried out by viewing under 254 nm UV light.

Mass spectra were obtained on an AEI MS-902 at 70 eV in the electron impact mode.

NMR spectra were recorded in $CDCl_3$ or $CDCl_3$-CD_3OD on a Bruker CXP300.

Spectral data for 11(S),13-dihydrolactucopicrin are as follows. UV (MeOH) absorbance maximum at 255 , 230 (sh) nm.

Mass spectrum (70 eV) *m/e* (relative intensity): 412 (M^+, 1), 322 (1), 260 (8), 231 (6), 198 (19), 187 (48) 152 (41), 107 (100).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.20 (H-13, d, J = 7 Hz), 2.32 (H-7, ddd, J = 11.5, 10.4, 10.3 Hz), 2.36 (H-9β, dd, J = 13.7, 2.5 Hz), 2.44 (H-15, s), 2.46 (H-11, dq, J = 11.5, 7 Hz), 2.70 (H-9α, dd, J =13.7, 10.7 Hz), 3.55 (H-α', d, J = 15 Hz), 3.58 (H-5, br d, J = 10 Hz), 3.61 (H-α'', d, J = 15 Hz), 3.69 (H-6, dd, J = 10.3, 10 Hz), 4.53 (H-14a, br d, J = 17.6 Hz), 4.82 (H-8, ddd, J = 10.8, 10.4, 2.5 Hz), 4.85 (H-14b, br d, J =17.6 Hz), 6.44 (H-3, m), 6.81 (H-3', H-5', d, J = 9 Hz), 7.13 (H-2', H-6', d, J = 9 Hz).

^{13}C-NMR (75 MHz, $CDCl_3$-CD_3OD) δ 14.5 (C-13), 21.2 (C-15), 40.5* (C-11), 40.8* (C-α), 44.5 (C-9) 48.8 (C-5), 58.5 (C-7), 61.9 (C-14), 70.6 (C-8), 80.6 (C-6), 115.5 (C-3'), 115.5 (C-5'), 123.8 (C-1'), 130.1 (C-2'), 130.1 (C-6'), 132.5# (C-1), 133.1# (C-3), 146.5 (C-10), 156.1 (C-4'), 171.0 (C-β), 172.8 (C-4), 177.0 (C-12), 195.0 (C-2).

CHAPTER 5

ENZYMATIC LIQUEFACTION OF CHICORY ROOTS: RELEASE OF SESQUITERPENE LACTONES

5.1 Introduction

The application of enzymes in the fruit industry has been described in literature for flavour enrichment of wine and fruit juices. In several fruits and fruit juices taste and aroma are associated with the presence of various free volatile monoterpene compounds (see for instance Wilson et al., 1984; Engel & Tressl, 1983). In addition to these free terpenes non-volatile water-soluble precursor compounds are present in these juices. In grape juice these precursors have been elucidated as a mixture of disaccharide glycosides of several monoterpene alcohols (Wilson et al., 1984). This was also found for passion fruit (Engel & Tressl, 1983), and papaya, apricot and mango (Salles et al., 1988). An extra enzymatic or acid hydrolysis of the fruit pulp or juice is necessary to obtain the volatile aglycons for improving the flavour (Wilson, et al., 1984; Engel & Tressl, 1983; Salles et al., 1988). The flavour of passion fruit juice was improved after treatment of the juice with ß-glucosidase; the press juice was used as substrate (Shoseyov et al., 1990). Monoterpene glycosides have a function in the biosynthesis of terpenes as reactants. They are also the transport form of the free monoterpenes in plants (Stahl-Biskup, 1987). Similar observations might be expected in the biosynthesis of sesquiterpene lactones in chicory roots.

Few publications are known in which the use of enzymes has been applied in order to increase the yield of flavours or antimicrobial compounds after partial maceration of plant material with (hemi)cellulase (e.g. Tateo and co-workers, 1979, 1977, 1982; Chuyen et al., 1982; Szakács-Dobozi et al., 1988). Nitz et al. (1985) incubated *Majorana hortensis* first with pectinase or glucosidase before extraction by steam distillation under reduced pressure. They compared these extracts with those obtained from untreated plant

material. Enzymatic treatment increased the yield of flavour compounds. This increase is probably due to precursors present in the plant as glycosides, which are released depending on the extraction method and the use of enzymes.

The reproducibility of the extraction of the bitter sesquiterpene lactones from chicory root was influenced by the history of the sample (Chapter 4). Storage and process conditions during drying and milling affected the yield of these compounds. Also the type of solvent used for the extraction, had an effect on the extraction efficiency.
In this Chapter the complete liquefaction of chicory roots with commercial enzyme preparations is described. These preparations are sold for their pectolytic and cellulolytic activities. The advantage of this process could be that more bitter compounds would pass into the liquid phase, than by a one step solid-liquid extraction. The liquefied root suspension should also contain inulin and other carbohydrates, which as a whole might be used as raw material in the manufacture of soft drinks.

5.2 Results and discussion

5.2.1 *Enzymatic liquefaction of chicory roots*

The release of lactucin, 8-deoxylactucin, and lactucopicrin has been studied during enzymatic liquefaction of chicory roots with commercial enzyme preparations, containing both pectinases and cellulases (see Fig. 5.1 for flow diagram). During 24 h of liquefaction, samples were taken and analysed by HPLC. The isocratic HPLC method was used, so no distinction between Lc, 8dLc, Lp and their dihydro derivatives was made. As control the same mixture was stirred without added enzymes. Both mixtures were kept at pH 4.
In the liquefaction mixture more Lc and 8dLc (and/or their dihydro analogues) could be detected compared to the control mixture. The total amount of sesquiterpene lactones investigated was rather constant for the control; in the enzyme treated sample the amount of lactucin and 8-deoxylactucin increased continuously over the investigated period (Fig. 5.2). No increase of lactucopicrin was found during enzymatic liquefaction of the roots.
Several hypotheses are formulated to explain the increase in bitter

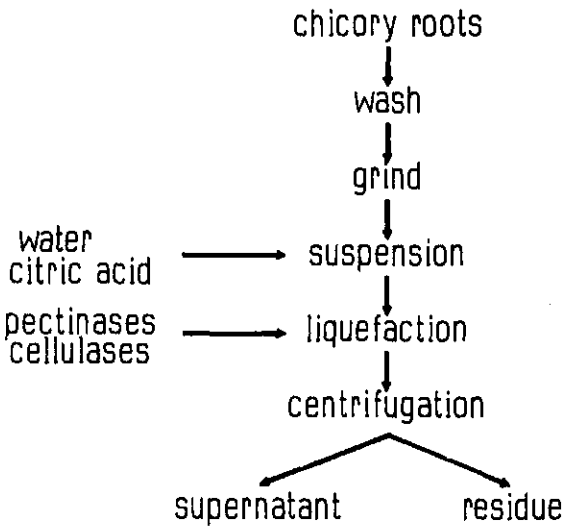


Fig. 5.1 Flow diagram for enzymatic liquefaction of chicory roots with commercial enzyme preparations.

constituents during enzymatic liquefaction.

Enzymatic hydrolysis of lactucopicrin into lactucin and p-hydroxy-phenylacetic acid using commercial enzyme preparations might partly explain the increase of lactucin during liquefaction. However, it was found that this reaction did not occur with the enzyme preparation used (Leclercq et al., 1988).

Unblanched chicory roots were used during enzymatic liquefaction. Therefore native chicory root enzymes could still be active during this liquefaction, and convert precursors into lactucin and 8-deoxylactucin.

On the other hand enzymes present in the used enzyme preparation might also be able to catalyse the formation of sesquiterpene lactones from precursors, e.g. glycosides. An unpurified commercial preparation consisting of many different activities was used for liquefaction of chicory roots. Prevalently pectolytic and cellulolytic activities were present, but also glycosidase activity might be expected in this preparation.

Therefore a chicory root extract with inactivated endogenous enzymes was used

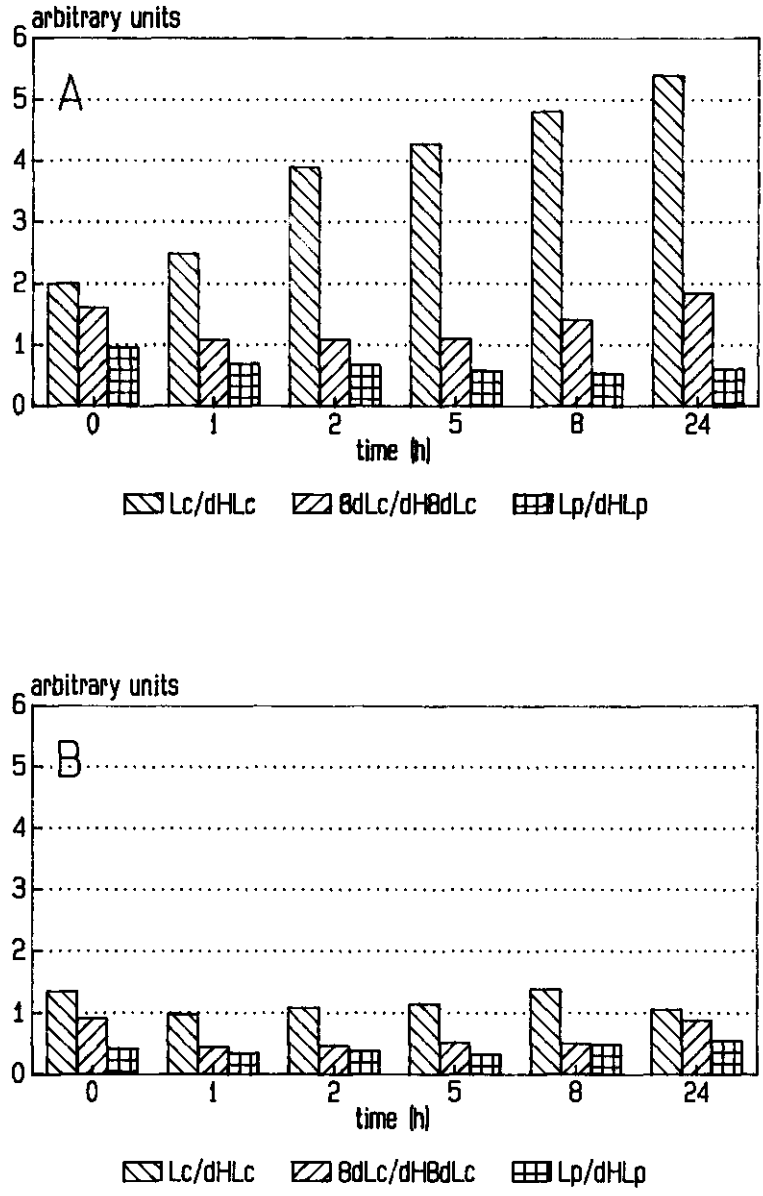


Fig. 5.2 Release of bitter compounds during enzymatic liquefaction of chicory roots (A) and control mixture (B).

as a substrate for a commercial enzyme preparation to elucidate, whether the increase of sesquiterpene lactones is due to the liquefaction process resulting in improved extraction efficiency, or to the conversion of precursors into known compounds (Section 5.2.2). All unknown compounds in the HPLC chromatogram, especially the polar compounds, were theoretically considered to be precursors.
The enzyme activity of chicory root was also investigated. Native enzymes might be active during liquefaction process, and thus be able to transform precursors into sesquiterpene lactones (Chapter 5.2.3).

5.2.2 *Enzymatic treatment of chicory root extract*

An aqueous chicory root extract (with inactivated endogenous enzymes) was incubated with Hemi II, a commercial enzyme preparation, having high pectolytic and cellulolytic activities. This solution was analysed qualitatively by gradient HPLC before and after enzyme treatment.
When compared with the control, some peaks in the chromatogram disappeared (peak N, T), or decreased (peak Q), while peaks of dHLc and dH8dLc increased (Fig. 5.3). Thus the commercial enzyme preparation is able to influence the composition of a chicory root extract. During enzymatic liquefaction of the chicory roots more polar compounds, which elute before peak N in the chromatogram, might also be converted into the known sesquiterpene lactones (dHLc, dH8dLc), and thus explain their increase. However, this was not further investigated.
With the above described experiment it is not possible to explain these results. Therefore the experiment was repeated with chicory root extract, which was partly purified on a small scale qualitatively with a Sep-Pak reversed phase C-18 column. The eight fractions obtained were treated with the same commercial enzyme preparation in a similar way as the whole extract, and analysed with HPLC before and after enzymatic treatment.
Peak N disappeared following enzymatic treatment and peak Q appeared (Fig. 5.4). In the fraction consisting of compound Q, peak Q decreased by enzymatic treatment, but did not disappear completely, while the peak of dHLc (peak 4) increased. The same was found in the fraction with compound T: peak T decreased, and the peak attributed to dH8dLc (peak 6) appeared in the HPLC

chromatogram after enzyme treatment. It was assumed that the molecular extinction coefficient did not change during enzymatic treatment, and thus that the decrease of for instance compound N is equal to the increase of compound Q.

No increase of Lp was seen after incubation of the chicory root extract with a commercial enzyme preparation.

As a result of above experiments a hypothesis is proposed: compound N (corresponding with peak N) is a precursor of compound Q (corresponding with

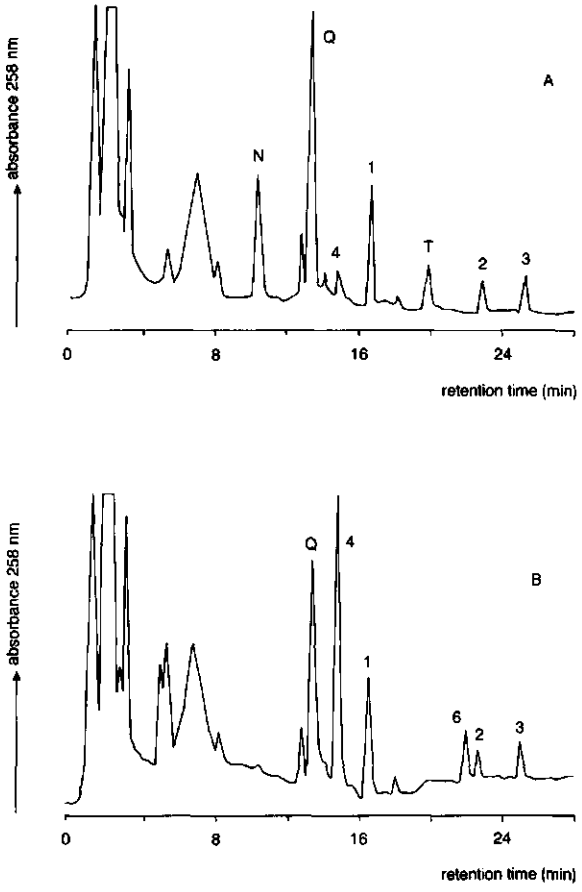


Fig. 5.3 HPLC chromatograms of chicory root extract before (A) and after (B) incubation with Hemi II enzyme preparation.
1=Lc; 2=8dLc; 3=Lp; 4=dHLc; 6=dH8dLc; Q=peak Q; T=peak T; N=peak N.

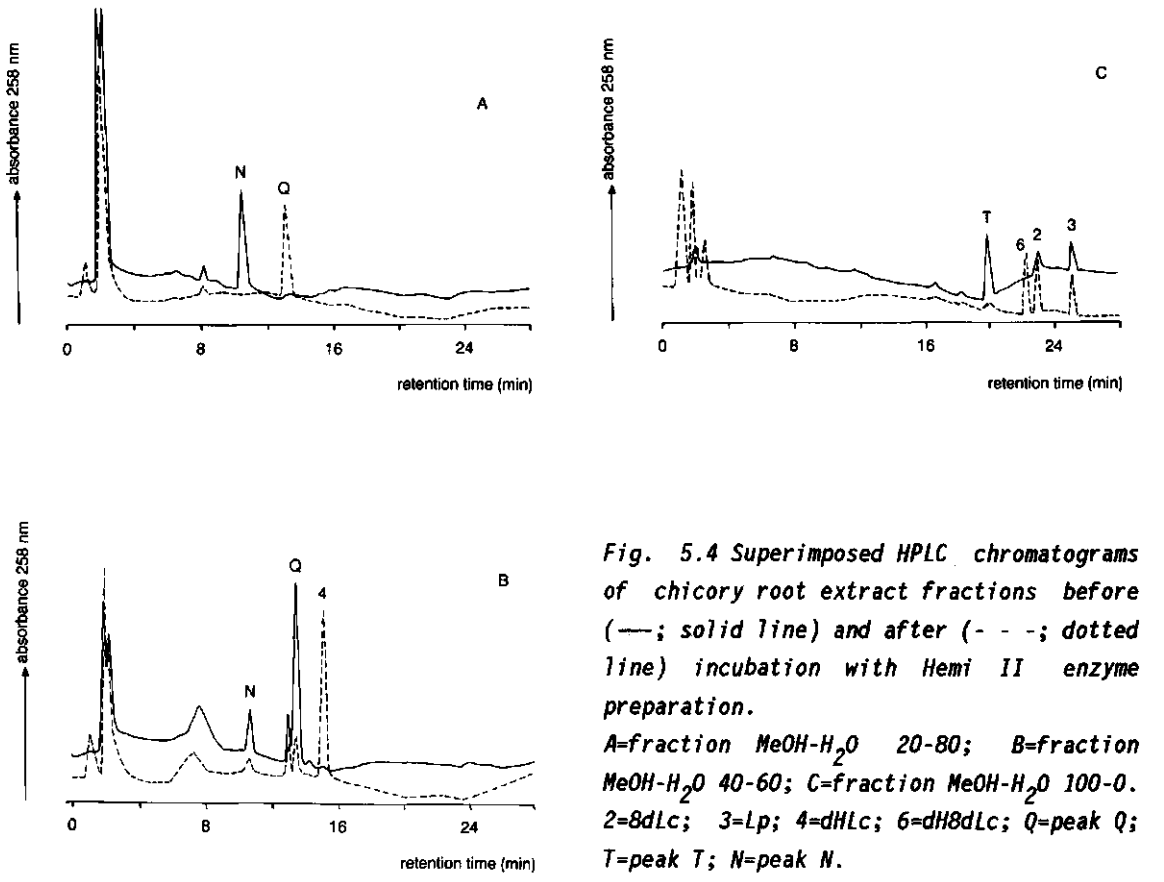


Fig. 5.4 Superimposed HPLC chromatograms of chicory root extract fractions before (——; solid line) and after (- - -; dotted line) incubation with Hemi II enzyme preparation.
A=fraction MeOH-H_2O 20-80; B=fraction MeOH-H_2O 40-60; C=fraction MeOH-H_2O 100-0. 2=8dLc; 3=Lp; 4=dHLc; 6=dH8dLc; Q=peak Q; T=peak T; N=peak N.

peak Q), compound Q is a precursor of dHLc, and compound T is the precursor of dH8dLc: N --> Q --> dHLc; T --> dH8dLc.

5.2.3 *Endogenous enzyme activity of chicory roots*

The experiment described in the previous Section was repeated with chicory root as the enzyme preparation. An aqueous chicory root extract (prepared according to Section 5.3.2 and thus free of active endogenous chicory enzymes) was incubated with a suspension of freeze dried chicory root powder. This crude enzyme preparation contained next to endogenous enzymes, also sesquiterpene lactones. This amount is neglected, because it will be far less than the amount of sesquiterpene lactones in the extract. The mixture was

incubated at 30 °C for 24 h (pH 6) and the changes in the extract were investigated by HPLC. The control was inactive chicory root powder, obtained by boiling the suspension for 8 min before it was added to the extract.
Compound Q and N decreased during incubation of the extract with a chicory root suspension, and Lc, dHLc, and dH8dLc increased. This suggests that native enzymes are able to form new compounds from an aqueous suspension of chicory root extract consisting of various known and unknown compounds detectable at 258 nm. Compound Q did not disappear completely after incubation with endogenous chicory enzymes, contrary to compound N which could not be identified after enzymatic treatment. Lp did not increase during enzymatic treatment with endogenous enzymes of the chicory extract.
These results are similar with those obtained by incubation of chicory root extract with commercial enzymes concerning decrease of compound N and Q, and increase of dHLc and dH8dLc. However, an increase of Lc is seen during incubation with endogenous enzymes.
A chicory root suspension incubated with inactive chicory root enzymes, did not change the composition of this extract. The amount of Lc, dHLc, and dH8dLc did not increase after incubation, and peak N and Q did not decrease.

Therefore the above mentioned hypothesis was extended. The enzymes from the chicory root as well as enzymes originated from a commercial enzyme preparation are able to convert precursors into other precursor compounds (compound N -> compound Q), and into known sesquiterpene lactones (compound Q -> dHLc; compound T -> dH8dLc). Chicory enzymes are able to convert the precursor(s) into Lc.
The fractions consisting of component Q and T were analysed and characterized in order to confirm this hypothesis.

5.2.4 *Identification of precursors*

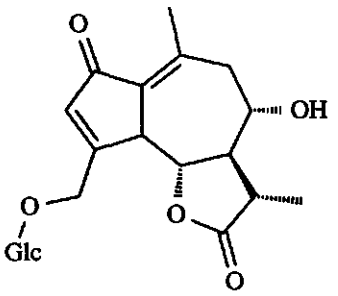
5.2.4.1 Compound Q

Various fractions of compound Q with different concentrations of compound Q were analysed for total sugar content according to Dubois et al. (1956). Also "bound" sugars (as glycosides) and sugars present as polymers were determined. It was found that a higher amount of compound Q present in the

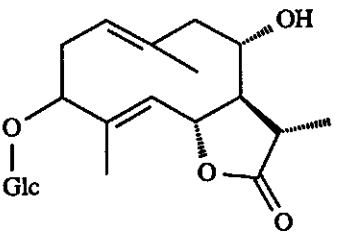
extract gave a more intense colour after reaction with phenol and sulphuric acid, which indicated that compound Q is indeed a glycoside. The sugar unit obtained after enzymatic hydrolysis of compound Q with Hemi II was found to be glucose as measured by HPLC.

Glycosides in chicory roots are already identified and described by Seto et al. (1988). They identified glycosides of sesquiterpene lactones of both the guaiane type, such as lactucin, and of the germacrane and eudesmane type not yet identified in our chicory root extract (Fig 1.1). In other Compositae, especially *Lactuca* sp., glycosides from Lc, dHLc, Lp, and 8dLc were found (Mahmoud et al., 1986; Nishimura et al., 1986a; Nishimura et al., 1986b; Adegawa et al., 1985).

Component Q was first analysed and characterized with ^{13}C-NMR. It was found that this compound consisted of two sesquiterpene lactone glycosides. Therefore this fraction was further purified with normal-phase HPLC. The ^{1}H-NMR, ^{13}C-NMR and MS spectra of the two fractions, Q1 respectively Q2, were compared with those of pure crepidiaside A, cichorioside B, cichorioside C, and sonchuside A. It was found that compound Q is a mixture of cichorioside B and cichorioside C. Cichorioside B is the glycoside of dHLc, and cichorioside C is a glycoside of a germacranolide (Fig. 5.5). Both components have also been identified in *Cichorium intybus* roots by Seto et al. (1988).



cichorioside B (Q1)



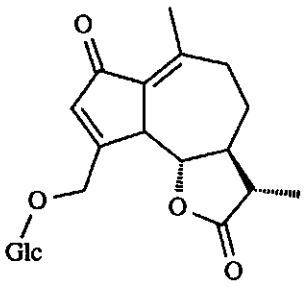
cichorioside C (Q2)

Fig. 5.5 Structures of compounds in peak Q.

5.2.4.2 Compound T

The fraction consisting of compound T was analysed and characterized by ^{1}H-NMR and ^{13}C-NMR. The data were compared with those of pure crepidiaside A, cichorioside B, cichorioside C, and sonchuside A, and with those published by Seto et al. (1988). They were found similar to the data of crepidiaside B, the glycoside of 11(S),13-dihydro-8-deoxylactucin (Fig. 5.6). This was confirmed by MS. The presence of crepidiaside B was reported in *C. endivia* and *C. intybus* by Seto et al. (1988).

For structure analysis Seto et al. (1988) and Adegawa et al. (1985) hydrolysed cichorioside B, cichorioside C respectively crepidiaside B with acid as well as with enzymes (cellulase respectively hesperidinase for 2 h at 38 °C; ratio enzyme-substrate was 1). Conversion of the glycosides was found into their respective aglycons and sugar unit. It might therefore be expected that above components are subjected to enzymatic hydrolysis during liquefaction of chicory roots.



crepidiaside B

Fig. 5.6 Structure of compound T.

5.2.5 *Conclusions*

The release of lactucin, 8-deoxylactucin and lactucopicrin during enzymatic liquefaction of chicory roots is described. More lactucin and 8-deoxylactucin are detected in the liquefaction mixture. No increase is observed in Lp

content during the liquefaction. However, analysis was carried out with the isocratic HPLC method, which can not distinguish Lc, 8dLc and Lp from their dihydro derivatives. Therefore the increase of Lc as seen during enzymatic liquefaction of chicory roots is due to an increase of Lc, dHLc, or both. The same can be stated for 8dLc and dH8dLc.

It was found that some commercial enzyme preparations, which are used for liquefaction because of their pectolytic and cellulolytic activities, are also able to hydrolyse cichorioside B and crepidiaside B, into their aglycons dHLc and dH8dLc respectively (Fig 5.7 and Fig. 5.8). The same phenomenon is observed when chicory root powder was used as crude enzyme preparation, indicating that chicory contains enzymes with glycosidase activity.

After incubation of purified chicory root fractions with commercial enzyme

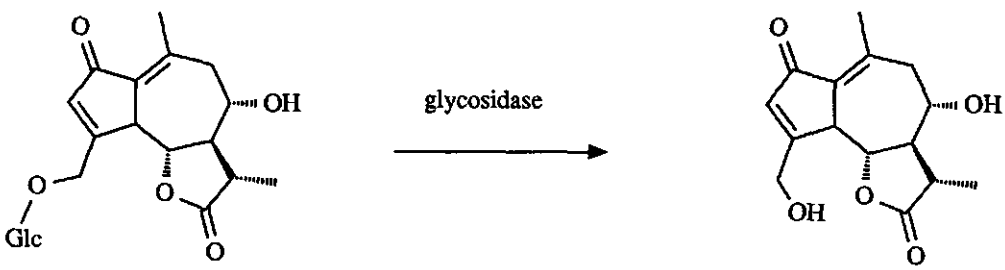


Fig. 5.7 Enzymatic conversion of cichorioside B into dHLc.

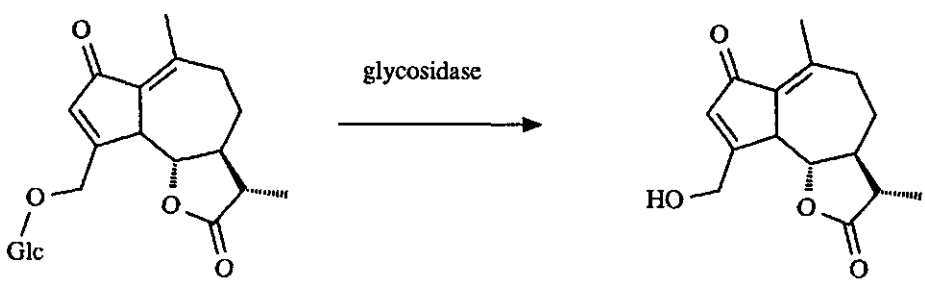


Fig. 5.8 Enzymatic conversion of crepidiaside B (compound 7) into dH8dLc.

preparation only an increase of dHLc and dH8dLc peaks is found in the gradient HPLC chromatogram. So the increase of lactucin and 8-deoxylactucin peaks in the isocratic HPLC chromatogram during enzymatic liquefaction of chicory roots is due to an increase of their dihydro analogue.
It is not unlikely that other polar compounds eluting at the beginning of the HPLC chromatogram are also involved in the enzymatic conversions during incubation of chicory roots with commercial enzyme preparations.
Compound N is converted into compound Q when incubated with a commercial enzyme preparation. The structure of compound N is not yet elucidated. However, the first results indicate that compound N is a disaccharide glycoside of dHLc.

In Chapter 4 the solvent extraction of sesquiterpene lactones from chicory roots is discussed. Storage conditions, milling, freezing, thawing, all influenced the total yield of these compounds. These observations can now be explained as a function of endogenous chicory enzyme activity, depending on the "history" of the sample. The quality, i.e. bitter taste of a chicory suspension after liquefaction, can therefore probably also be affected by the type of enzyme used for liquefaction and by the activity of endogenous enzymes from the roots. This will be discussed further in Chapter 8.

Compound Q appeared to be a precursor of dHLc. It consists of two compounds: cichorioside C, a glycoside of a germacranolide, and cichorioside B, the glycoside of dHLc. Also crepidiaside B is identified in chicory roots. This compound is the glycoside of dH8dLc. Both cichorioside B and crepidiaside B can be hydrolysed into their sugar unit and respective aglycons. These conversions have now been proven to occur during enzymatic liquefaction of chicory roots. Crude chicory enzymes are also able to hydrolyse cichorioside B and crepidiaside B.
The hydrolysis of cichorioside C into its (unnamed) aglycon and sugar unit has yet not been proven during enzymatic liquefaction of chicory roots, but it is not unlikely.
It is yet not known whether commercial enzyme preparations consisting of many activities are able to convert a germacranolide into a guaianolide, for instance cichorioside C -> cichorioside B. Probably the pH plays also a role in this conversion. New experiments are necessary to verify both options.

Although still not proven, it is plausible that chicory enzymes are also able to perform the above reaction. Both types of sesquiterpene lactones are characteristic for the chicory roots. Many endogenous enzymes are involved in their synthesis. It is thus not unlikely that endogenous enzymes are able to catalyse the reaction cichorioside C -> cichorioside B. This may be a subject for further investigations.

5.3 Experimental section

5.3.1 *Materials*

Fresh chicory roots were obtained from a grower in Veenendaal, the Netherlands. The roots were used as such, or first frozen (-30 °C), freeze dried, and crushed by sieving (4 mm). The powder was stored at 4 °C in dark. Before use the chicory roots were first washed to remove adhering soil.

Crepidiaside A, cichoriòside B, cichorioside C, and sonchuside A, were a gift from T. Myase, School of Pharmaceutical Sciences, University of Shizuoka, Japan.

5.3.2 *Preparation of chicory extract*

Freeze dried chicory root powder (4.5 g) was soaked (15 min) with twice the natural quantity (60 g) of water added to the dried powder. This was extracted with ethyl acetate by boiling twice under reflux with a fresh portion of solvent for 15 min. The water layer and EtOAc layer were separated (Fig. 4.5). The chicory root powder was removed from the water layer by filtration, and the residue resuspended in water (60 g), and a second extraction with EtOAc as described above, was carried out. The two water layers were combined. The remaining EtOAc was removed by evaporation. The final volume of the water extract was 100 ml. Endogenous chicory root enzymes were inactivated during this process.

5.3.3 *Enzymatic treatment with commercial enzyme preparation*

Portions of fresh chicory roots (150 g) were suspended in 150 ml of 22 mM citric acid, to which 150 mg of Rapidase C600 (pectolytic and cellulolytic enzymes (Gist-brocades, the Netherlands)) was added as a dry powder. The suspension (pH 4) was stirred continuously at 40 °C. After various periods of time (0, 1, 2, 5, 8 and 24 h) samples were removed with a pipette with a wide orifice (9 mm I.D.) and centrifuged for 30 min at 10000 g to obtain residue (pellet) and supernatant (liquid phase). The control was the same mixture without added enzymes but using 73 mM citric acid instead of 22 mM to maintain the pH at 4, since no galacturonic acid is liberated.
The supernatant obtained after liquefaction of the roots (as well as of the control mixture) was diluted with methanol (1-1 v/v) and after filtration of the suspension injected into the liquid chromatograph for determination of Lc, 8dLc and Lp. The isocratic HPLC method was used as described in Section 4.4.3.
From the solid material, i.e. residue obtained after liquefaction or solid material obtained from incubation mixture without enzymes, the sesquiterpene lactones were extracted with chloroform as described in Section 4.4.2. The obtained extract was injected into the liquid chromatograph. Similar equipment and procedures were used as described for the supernatant. With this HPLC method Lc, 8dLc, and Lp could not be distinguished from their dihydro derivatives.

Chicory root extract (prepared as decribed in Section 5.3.2) was incubated with enzyme preparation Hemi II (Gist-brocades, the Netherlands), consisting of hemicellulolytic, cellulolytic and pectolytic activities (Militz, 1990). Before use this enzyme was purified by dialysis in order to decrease the sugar content (the enzyme preparation contains sugar as drying aid). The enzyme concentration used was 50 mg enzyme/10 ml 0.05 M sodium acetate buffer, pH 5. Incubation of the sample (250 μl in 750 μl 0.05 M NaOAc buffer, pH 5) was carried out at 30 °C (water bath) during 6 min with 50 μl of Hemi II suspension; final volume was 1050 μl. The enzymes and extract were mixed thoroughly before incubation. After incubation the enzymes were inactivated by heat treatment (water bath, 100 °C, 10 min). Blank was 250 μl sample dissolved in 800 μl buffer solution.

Chicory root extract was obtained as described in Section 5.3.2. This extract was fractionated on a Sep-Pak RP-18 column (10 x 5 mm) (Waters Assoc.). A sample (1 ml) was fractionated by gradient elution (100 % H_2O to 100 % MeOH) in 8 steps of 1 ml each. The 8 fractions obtained were incubated with Hemi II at pH 5: a 200 μl sample was treated with 50 μl of enzyme in 750 μl NaOAc buffer (0.05 M) at 30 °C during 10 min. The enzymes were inactivated by heat treatment after incubation (100 °C, 10 min). The control experiment was carried out with 250 μl sample and 750 μl 0.05 M NaOAc buffer at pH 5.

5.3.4 *Chicory root as enzyme preparation*

Chicory root extract was prepared according to Section 5.3.2. This substrate (2 ml) was diluted with buffer (pH 6; 17.5 ml) made according to McIlvaine (1921). The total mixture was incubated with 0.5 ml of an aqueous suspension of freeze dried chicory root powder (4% (w/v)) at 30 °C for 24 h. After incubation the enzymes were inactivated by boiling, and the samples were analysed by gradient HPLC.
A control experiment was carried out by dissolving the chicory root powder in boiling water (100 °C, 8 min) to inactivate endogenous chicory enzymes. After cooling, the incubation and analysis was carried out as described above.

5.3.5 *Isolation and purification of compound Q*

Fresh chicory roots (7.5 kg) were extracted twice with 10 l of MeOH-H_2O (80-20) under reflux for 20 min. The two extracts were combined and concentrated under reduced pressure until all MeOH was removed. The combined extract (5.3 l) was partitioned twice with BuOH (1.7 resp. 2.3 l). The BuOH fraction was concentrated under reduced pressure, and 64.18 g of residue was obtained. This fraction was washed twice with MeOH. The obtained MeOH solubles (42.10 g) were fractionated by gelfiltration using a Jobin Yvon 500 x 40 mm Modulprep column filled with 300 g of Sephasorb HP ultrafine (Pharmacia), the eluent was MeOH-H_2O (80-20), the flow rate was 10 ml/min, UV detection at 254 nm was used. Fractions (5-20 ml) were collected and analysed by gradient RP-HPLC as described in Section 4.4.3. The fractions consisting mainly of

compound Q and compound T were combined (total 1.09 g) and separated with RP-MPLC and RP-HPLC respectively (RP-MPLC: a Jobin Yvon 500 x 20 mm Modulprep column filled with 60 g Spherisorb C-18, particle size 40 μm, flow rate 5 ml/min, detection at 254 nm, eluent MeOH-H_2O (50-50); RP-HPLC was carried out on a 250 x 10 mm column filled with Microsorb C-18, particle size 5 μm (Rainin C-18, 80-299-C5, s/n 10027), flow rate 4 ml/min, detection at 258 nm, eluent MeOH-H_2O (30-70)). Pure compound Q (13.9 mg) and compound T (13.3 mg) were obtained.

After analysis of compound Q with ^{13}C-NMR it was apparent that compound Q consisted of two sesquiterpene lactones. Therefore compound Q was dissolved in BuOH and separated and purified with normal-phase HPLC (Microsorb silica gel column, particle size 3 μm, flow rate 1 ml/min, eluent tBuMeO-MeOH (90-10)). The compounds were detected by their UV absorbance at 215 and 258 nm. Two fractions were obtained, consisting of cichorioside B (3.0 mg) respectively cichorioside C (3.1 mg), were investigated by ^{1}H-NMR and MS. The obtained spectra were compared with those of Seto et al. (1988).

5.3.6 *Isolation and purification of compound T*

Pure compound T (13.3 mg) from fresh chicory roots (7.5 kg) was obtained as described in Section 5.3.5. It was characterized with ^{1}H-NMR, ^{13}C-NMR and MS. The structure was found to be crepidiaside B. The obtained spectra were compared with those described by Seto et al. (1988).

5.3.7 *Analysis*

EtOAc extracts were analysed by HPLC as described in Section 4.4.3. However, a different gradient elution pattern was used. Eluent A was water, eluent B was MeOH. In 26 min 90% eluent A decreased to 20%, and 10% eluent B increased to 80%. Flow rate was 1.5 ml/min. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in D_2O on a Bruker CXP300 (200MHz and 50 MHz respectively).

Mass spectra were obtained on an AEI MS-902 at 70 eV in the electron impact mode.

The total amount of sugar present in the fractions containing only compound Q

were analysed according to Dubois et al. (1956). Glucose was used for calibration.

HPLC analysis of sugars was carried out using a CHPB column (Merck) connected in line with a guard column filled with 35% AG50-WX4 (minus 400 mesh, H^+-form) and 65% AG3-X4A (200-400 mesh, OH^--form) both from Bio-Rad. Eluent was double distilled water, degassed by filtration through a Millipore-filter of pore size 0.45 μm for aqueous solutions. A RI (refractive index) detector was used. The column and detector were kept at 85 and 40 °C respectively. The flow rate was 1 ml/min. Before analysis 1 ml of sample was treated with 50 μl of 1 M $Pb(NO_3)_2$ to avoid precipitation in the column.

CHAPTER 6

ENZYMATIC LIQUEFACTION OF CHICORY ROOTS: ROLE OF ENDOGENOUS ENZYMES

6.1 Introduction

During enzymatic liquefaction of chicory roots glycosides of known sesquiterpene lactones are hydrolysed into their aglycons and carbohydrate unit. The added commercial enzyme preparation as well as endogenous enzymes from chicory are likely to be responsible for this phenomenon. There are also indications that germacrane type sesquiterpene lactones are converted into guaiane type during enzyme treatment (see Chapter 5).
The use of isolated enzymes from plants to generate interesting secondary metabolites (for instance flavours) is still in its infancy (Drawert, 1988). Plant enzymes are only used in cell and tissue cultures, but to date industrial attempts to establish these techniques for the production of biochemicals have been hampered by low yields of the desired substances (Constable, 1988).

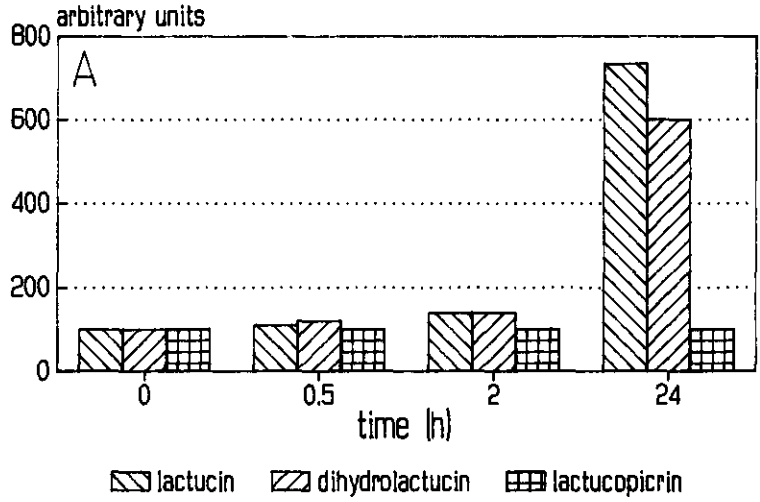
The only publications on chicory enzymes known to the author are those about enzymes involved in the fructose metabolism in chicory root. Singh and coworkers investigated glucofructosan metabolism and isolated also fructosyl transferase, the enzyme responsible for the synthesis of fructosan (inulin) (Singh & Bahtia (1971a,b); Gupta et al. (1986)).
Other enzyme systems of this plant have not been reported so far.
Chicory roots contain interesting enzymes (see Section 5.2.3). Therefore endogenous chicory root enzymes were studied in more detail. The release of sesquiterpene lactones during incubation with chicory root powder as enzyme preparation was investigated. Chicory root extract was used as substrate. The properties of chicory root enzymes obtained after salt extraction of the chicory powder, and after further purification were also studied, and

compared with commercial enzyme preparations.

6.2 Results and discussion

6.2.1 *Release of sesquiterpene lactones*

Enzymes from chicory roots can convert precursor compounds into known sesquiterpene lactones, without any other enzymes involved (see Section 5.2.3). This indicates that endogenous enzymes from the roots might also be responsible for the increase of lactucin during liquefaction. The experiment as discussed in Section 5.2.3 was carried out for only one incubation time (24 h). Chicory root extract (free of active endogenous enzymes) was used as substrate and freeze dried chicory root powder as the enzyme preparation.
The release of Lc, dHLc, and Lp was investigated with chicory root enzymes during incubation, and compared the results with those obtained from enzymatic liquefaction of the roots with commercial enzymes (described in Section 5.2.1). During 24 h samples were taken and analysed by gradient HPLC. The control with inactive chicory root enzymes was carried out simultaneously.
An increase in Lc and dHLc is seen during incubation of chicory root extract as substrate and chicory root powder as crude enzyme preparation (Fig. 6.1). This increase is significant compared with the inactivated control. Also an increase is observed for dH8dLc during incubation with chicory root compared with control (data not shown). The amount of Lp remains constant over the period investigated. In the control mixture Lc and Lp increased somewhat, but this might be due to a solubility effect.
Precursors of Lc, dHLc and dH8dLc are hydrolysed by chicory enzymes into the sugar moiety and aglycons. The precursors of dHLc and dH8dLc are cichorioside B respectively crepidiaside B. The glycoside of Lc is expected as precursor in view of the results above mentioned. This compound, picriside A (Fig. 6.2) is to date only identified in *Picris hieracioides* L., like chicory also a member of the Compositae family (Nishimura et al., 1986b). The increase of Lc during enzymatic treatment with chicory enzymes proves indirectly that picriside A is a constituent of chicory roots. However, the best experiment



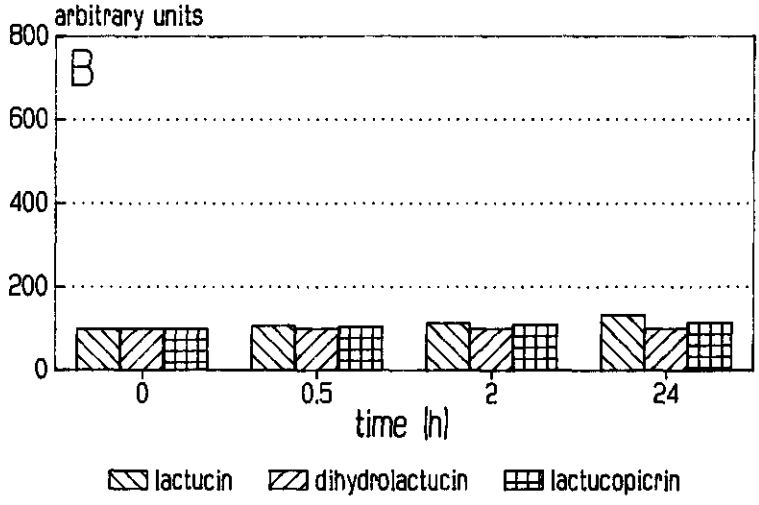


Fig. 6.1 Increase of sesquiterpene lactones during incubation of chicory root extract with crude chicory root enzyme preparation (A) and control (B).

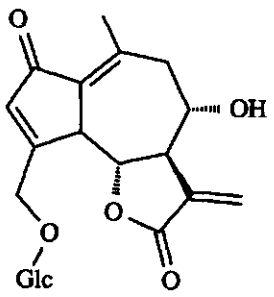


Fig. 6.2 Structure of picriside A.

in this matter is extraction and identification.

Above results confirm our earlier findings using commercial enzyme preparations and crude chicory enzymes (see Chapter 5). The increase of Lc during incubation with enzymes is similar, compare Fig. 5.1 with Fig. 6.1. However, the increase of Lc as shown in Fig. 5.1 is in fact an increase of Lc, dHLc or both. This increase seems less compared to the increase of Lc and dHLc as shown in Fig. 6.1. The differences found in the two experiments are due to different substrates and different enzymes. The experiment described in Section 5.2.1 (see Fig. 5.1) was carried out with fresh chicory root as substrate, and this new experiment with chicory root extract.

In the extract only precursors were available as substrate for the endogenous enzymes. During the liquefaction of chicory, also cell wall components are present in a higher amount than the precursors of the sesquiterpene lactones. The used commercial enzyme preparations are selected for hydrolysing the plant cell wall and not precursors.

Additional the experiments with commercial enzymes were carried out using a different extraction procedure for roots and pellet: chloroform was used as solvent instead of EtOAc. This should influence negatively the results obtained with liquefaction with exogenous enzymes. A less specific isocratic HPLC method was used in the experiments as described in Section 5.2.1; the

above mentioned results were obtained with the gradient HPLC method described in Section 4.4.3.

When incubation with endogenous enzymes is continued (up to 3 weeks), only Lc, dHLc, and dH8dLc are found in the chicory root extract. This indicates that endogenous enzymes seem also to be able to convert germacranolide precursor into a guaianolide. All other constituents of the chicory root extract, polar as well as apolar (Lp), have thus been used as substrate.

6.2.2 *Enzyme characteristics*

6.2.2.1 pH and temperature optimum

The effect of pH (pH 3-8) on the release of three bitter sesquiterpene lactones by endogenous chicory root enzymes was investigated (30 °C, 24 h). Chicory root extract free from endogenous enzyme activity, was used as substrate. The results are shown in Fig. 6.3.

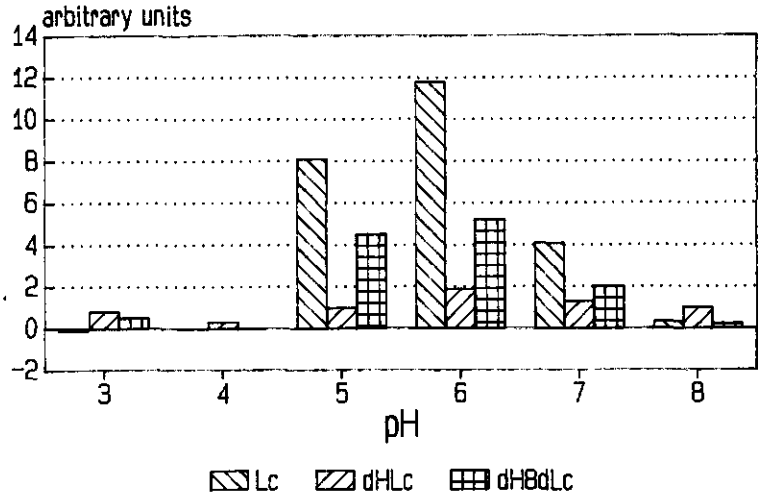


Fig. 6.3 Effect of pH on the release of sesquiterpene lactones during incubation of chicory root extract with crude chicory enzyme preparation.

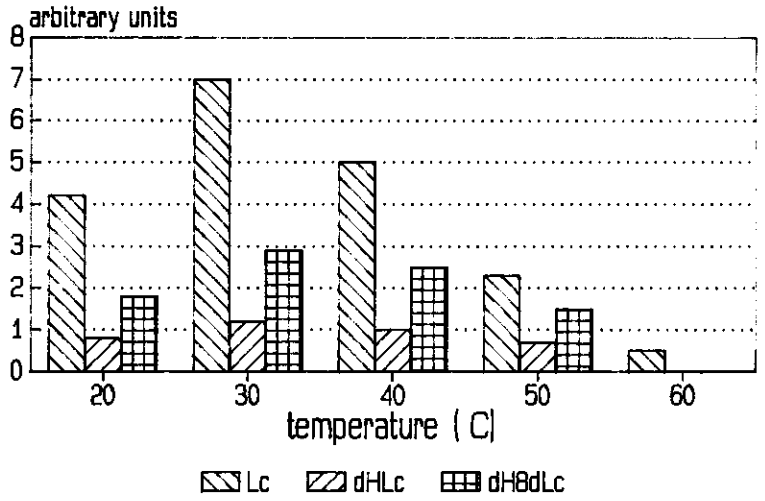


Fig. 6.4 Effect of temperature on the release of sesquiterpene lactones during incubation of chicory root extract with crude chicory enzyme preparation.

For Lc, dHLc as well as dH8dLc the pH optimum was between pH 5 and 6. Hardly any chicory enzyme activity could be detected at pH 3-4 and 8. The rate at which the hydrolysis of precursors into the sesquiterpene lactones investigated occurred, was higher at pH 6 than at pH 5. So all further experiments were carried out at pH 6.

The effect of temperature on the formation of sesquiterpene lactones when incubated with a crude chicory root enzyme preparation at pH 6, is illustrated in Fig. 6.4. In this experiment chicory root extract was also used as substrate. The optimum temperature giving maximum release for all sesquiterpene lactones investigated, is 30 °C. The temperature used during enzymatic liquefaction of chicory roots with commercial enzyme preparations (40 °C) is not optimal for the chicory root enzymes. Especially the formation of Lc has been decreased.

6.2.2.2 Conversion of compound N and compound Q

The conversion of precursors, especially compound N and compound Q (consisting of cichorioside B and cichorioside C) was investigated during incubation of chicory root extract (free of endogenous enzyme activity) with chicory root powder as crude enzyme preparation for 24 h at 30 °C and pH 6.

Fig. 6.5 shows the pH optimum for conversion is pH 6 for compound N; no pH optimum could be detected for compound Q. This might be due to the character of compound Q, which consists of two glycosides: cichorioside B and cichorioside C, which are both different sesquiterpene lactone type (guaiane respectively germacrane). The hydrolysis of compound Q into its aglycons may need at least two enzymes with different properties.

Other precursor compounds, among them crepidiaside B, also decrease after incubation of an chicory extract with crude chicory root enzymes (data not shown).

For endogenous chicory root enzymes the optimum conditions for the conversion

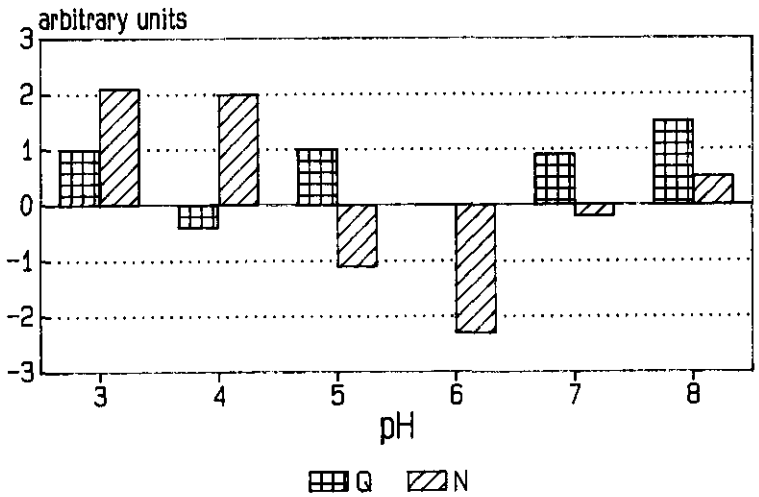


Fig. 6.5 Effect of pH on the decrease of compound Q (cichorioside B, cichorioside C) and compound N during incubation of chicory root extract with crude chicory root enzyme preparation.

of precursors into known sesquiterpene lactones are: pH 6 and 30 °C. The rate of these reactions is less than that of commercial enzyme preparations. After 2 h incubation with chicory root enzymes hardly any difference between the incubation mixture and control mixture is seen, contrary to the commercial enzymes. The optimum pH and temperature for the commercial preparation is pH 4 and 40 °C (Chapter 5). These conditions were used during liquefaction. At pH 4 hardly any endogenous chicory enzyme activity is seen, and also 40 °C is suboptimal for the chicory enzymes. Thus the release of sesquiterpene lactones during enzymatic liquefaction is only due to the commercial enzymes used.

6.2.2.3 Purification of chicory enzyme

Freeze dried chicory root powder was extracted with a solution of salt. The extract was dialysed and further purified by ion exchange and ultra filtration as described in Section 6.4.2. Five fractions were obtained and investigated for the conversion of sesquiterpene lactones and their precursors. Chicory root extract was used as substrate (pH 6, 24 h).

Similar results were obtained as described in Sections 6.2.2.1 and 6.2.2.2: a decrease of compound N and cichorioside B, and an increase of Lc, dHLc and dH8dLc was found.

One of these fractions was found to have the highest enzyme activity for conversion of compound N and cichorioside B into other components, among them dHLc. This compound was increased after incubation.

This purified chicory root enzyme fraction was compared with crude chicory enzyme and two commercial enzyme preparations for their ability to hydrolyse pure cichorioside B and crepidiaside B into dHLc respectively dH8dLc. The two commercial enzyme preparations, Exo I and Hemi II, had similar p-nitrophenyl-β-D-glucopyranoside (PNPG) activity, which was used as standard.

Exo I is an enzyme preparation with cellulase (mainly β-glucosidase) activity, and Hemi II is a preparation consisting of (hemi) cellulase and pectinase activities. The results are summarized in Table 6.1.

After 21 h incubation with Exo I all cichorioside B and crepidiaside C were hydrolysed into dHLc and dH8dLc respectively. This could not be reproduced

using the other enzyme preparations, only partial hydrolysis was seen. With inactive chicory enzyme no hydrolysis of cichorioside B and crepidiaside B was found.

From Table 6.1 it can be concluded that Exo I has a higher enzyme activity, expecially ß-D-glucosidase, than Hemi II, relating to hydrolysis of cichorioside B and crepidiaside B, despite their similar glucosidase activity

Table 6.1 Enzymatic hydrolysis of cichorioside B (Q) and crepidiaside B (T) after incubation for 21 h with enzyme preparations. Data are in height (mm) of the compounds in the HPLC chromatogram.

	compound			
Enzyme preparation	Q	dHLc	T	dH8dLc
Blank	36	-	80	-
Exo I	-	33	-	74
Hemi II	17	19	32	46
Crude chicory enzyme	12	20	13	63
Purified chicory enzyme	22	12	54	26

towards PNPG. This might be due to enzyme activities other than ß-D-glucosidases, which are necessary for the conversion of precursors into the compounds investigated.

According to Günata et al. (1990) the activity of ß-glucosidases is dependent on the structure of the aglycon (primary, secondary, or tertiary alcohol), and the origin of the enzyme. However, cichorioside B and crepidiaside B are both glycosides of a primary alcohol, so this can not explain differences

between Exo I and Hemi II.
The crude chicory root enzyme preparation hydrolysed more cichorioside B and crepidiaside B than the purified chicory enzyme: only 40% of both substrates were hydrolysed by the purified enzyme preparation, contrary to the crude chicory enzyme preparation, which hydrolysed about 70-80% of the substrates. The activity of the crude enzyme preparation towards crepidiaside B was higher than that towards cichorioside B. So during purification of the chicory enzyme part of the enzyme activity with respect to the hydrolysis of both pure compounds has been partly lost. Hemi II had similar activity for cichorioside B and crepidiaside B as had the purified chicory root enzyme preparation, however, the rate of hydrolysis was higher when Hemi II was used at the time scale investigated.

6.3 Conclusions

Endogenous chicory root enzymes are able to hydrolyse precursor compounds into their aglycon and sugar unit, as can be seen by the increase of dHLc and dH8dLc and the decrease of cichorioside B and crepidiaside B. Under the conditions used, more time is necessary to hydrolyse the precursor glycosides with endogenous enzymes than with the commercial enzyme preparations.
Endogenous chicory enzymes are also able to convert germacrane type of compounds into guaiane type of compounds. Only guaianolides (Lc, dHLc, and dH8dLc) are found in chicory extract after incubation with these enzymes. No Lp could be detected. This compound is probably hydrolysed to Lc and p-hydroxy-phenylacetic acid by endogenous enzymes.
The pH optimum of these enzymes is pH 6, and the optimum temperature is 30 °C. Enzymatic liquefaction with commercial enzymes was carried out at pH 4 at 40 °C. Under these conditions endogenous chicory root enzymes are not active. So the release of sesquiterpene lactones as described in Chapter 5 is therefore due to the added, exogenous commercial enzymes.
The enzyme preparation Exo I has a high activity for hydrolysing pure cichorioside B and crepidiaside B into dHLc respectively dH8dLc. Hemi II has a lower activity towards these two compounds despite similar glucosidase activity for PNPG.

The purified chicory root enzyme fraction has lost part of the enzyme activity in comparison with the crude chicory enzyme preparation, since it is less able to hydrolyse crepidiaside B.

6.4 Experimental section

6.4.1 *Materials*

Pure cichorioside B and crepidiaside B were obtained from fresh chicory roots as described in Section 5.3.4 and 5.3.5.

6.4.2 *Release of sesquiterpene lactones*

Chicory root extract was prepared as described in Section 5.3.2. It was free of endogenous chicory enzyme activity. This substrate (2 ml) was diluted with McIlvaine buffer (17.5 ml, pH 6). The total mixture was incubated (30 °C) with 0.5 ml of an aqueous suspension of freeze dried chicory root powder (4% (w/v)). After various periods of time (0, 0.5, 2 and 24 h) samples (0.5 ml) were removed. Enzyme inactivation was by boiling (water bath, 8 min). The extracts were analysed by HPLC as described in Section 4.4.3. Compounds were identified according to their retention time.
A control experiment was carried out by dissolving the chicory root powder in boiling water (100 °C, 8 min) to inactivate the endogenous chicory enzymes. After cooling, the incubation and analysis were carried out as described above.

6.4.3 *pH and temperature optimum*

Buffers of pH 3-8 were prepared according to McIlvaine (1921). Incubation conditions were: 50 µl enzyme, 200 µl substrate, 1750 µl buffer solution at 30 °C for 24 h. Chicory root extract was obtained as described in Section 5.3.2, and used as substrate. Inactivation was by boiling for 8 min. The

samples were analysed by gradient HPLC as described in Section 4.4.3. In the control mixture, the enzyme was replaced by the same amount of buffer.

In order to determine the temperature optimum of chicory root enzymes, 50 μl enzyme was mixed with 200 μl substrate and 750 μl McIlvaine buffer (pH 6), and incubated (20-60 °C) for 2 h. The mixture was boiled for 8 min to inactivate the enzyme. Samples were analysed by gradient HPLC.

6.4.4 *Purification of chicory root enzymes*

Freeze dried chicory root powder (6 g) was extracted with 5% NaCl solution (81 ml), containing also 0.25% ascorbic acid and 0.01% sodium azide, for 2 h at pH 7. During extraction the material was stirred continuously and the temperature kept constant at 5 °C. After centrifugation (10 min, 12000 g) the solution was dialysed twice using 5 l sodium succinate solution (20 mM, pH 6.8). This crude enzyme solution was used for the experiments with pure cichorioside B and crepidiaside B.

A part of above enzyme solution was further purified by fractionation with DEAE (Biogel) ion exchange column (22 x 3.5 cm). Before elution the enzyme solution (35 ml) was first centrifuged (10 min, 49500 g) to remove the final remainder of chicory root particles.
The enzymes were eluted from the column at 5 °C with 20 mM sodium succinate (pH 6.8) with gradient of NaCl (0-1 M) as eluent. Gradient was 100% 20 mM sodium succinate (A) for 2 h; linear increase for 6 h from A to 100% A with 0.5 M NaCl (B); elution with B for 2 h; linear gradient from 100% B to 100% A with 1 M NaCl in 4 h. Flow rate was 20 ml/h, UV detection at 280 nm was used. Fractions (300 each of 10 ml) were collected. The fractions obtained were assessed for p-nitrophenyl-ß-D-glucopyranoside (PNPG) activity, on the basis of which they were combined into 5 fractions.

6.4.5 *Enzymatic hydrolysis of cichorioside B and crepidiaside B*

Solutions of pure cichorioside B and crepidiaside B (1950 µl; 0.1 mg/100 ml resp. 0.3 mg/100 ml) were incubated with 50 µl of Exo I, Hemi II, crude chicory root enzyme preparation, and purified chicory root enzyme preparation (made as described in Section 6.4.4) at pH 5 (Exo I, Hemi II) or pH 6 (chicory root enzyme preparations) for 21 h at 30 °C. Samples were taken before and after incubation and analysed with gradient HPLC after inactivation of the enzymes by boiling (water bath, 8 min).

6.4.6 *PNPG activity*

Enzyme fractions were analysed for their p-nitrophenyl-ß-D-glucopyranoside activity. Chicory root enzyme (75 µl) was incubated with 50 µl PNPG solution (0.375%, McIlvaine buffer, pH 6) for 45 min at 30 °C. The reaction was terminated by addition of 125 µl 0.5 M glycine buffer (pH 9) containing 0.002 M EDTA. The extinction of the solution was measured at 405 nm, after which PNPG activity could be calculated.

CHAPTER 7

ENZYMATIC LIQUEFACTION OF CHICORY ROOTS: RELEASE OF INULIN

7.1 Introduction

In addition to bitter sesquiterpene lactones chicory roots contain also inulin, which is a carbohydrate reserve. Inulin cannot be digested by humans because of lack of the appropriate enzymes, and may therefore be used as dietary fibre. Inulin can be used as raw material for the production of fructose syrup (Zittan, 1981) and 5-hydroxymethylfurfural (Kuster, 1989).
Inulin is usually extracted from sliced plant material using hot water. The yield of inulin depends on its degree of polymerisation, and on the milling degree of the chicory roots from which inulin has to be extracted (Fleming & Groot Wassink, 1975).
De Baynast de Septfontaines et al. (1986) have patented a process for liquefaction of sugar beets by an enzymatic treatment using cellulases or pectinases, and inulinases, which is also suitable for chicory roots. The resulting hydrolysate may be used for fermentation to produce alcohol. The advantage of this process over extraction by diffusion is that no extra water is needed during incubation, and that it is suitable for two types of agricultural raw materials. There are no claims for a higher efficiency of the process compared to hot water extraction.

Enzymatic liquefaction of chicory roots with pectinases and cellulases should not only release the bitter constituents but also inulin, and optionally after treatment with inulinase, fructose. Thus a bitter, sweet syrup can be obtained, which might be used as raw material for the production of beverages, e.g. tonic water.
Release of inulin was investigated as well as inulinase activity of enzyme preparations used.

7.2 Results and discussion

7.2.1 *Enzymatic liquefaction of chicory roots*

Several commercial enzyme preparations were evaluated for their ability to liquefy chicory roots and to release inulin without its hydrolysis by determination of the total sugar content and reducing sugars in the supernatant after liquefaction.

A large amount of fructose and other reducing sugars was found in the supernatant after liquefaction of chicory roots using Rapidase C80 (see Table 7.1). This is due to the inulinase activity of this enzyme preparation, contrary to Maxazym CL2000 and Rapidase C600, which both gave higher amounts of inulin. However, Maxazym CL2000 did not liquefy chicory roots very well: giving only 53% of total sugars in the supernatant. With a mixture of

Table 7.1 Sugar composition of supernatant after enzymatic liquefaction of chicory roots with several enzyme preparation at pH 4 for 24 h. Data are expressed as % of total dry matter of chicory roots.

enzyme preparation*	enzyme concentration**	total sugars	glucose	fructose	sugars with DP≥4
C80	0.25	63	13	43	0.2
CL2000	0.20	53	2	2	3.8
C80 + CL2000	0.25 + 0.20	64	16	38	0
C80 + CL2000	0.20 + 0.20	64	16	36	0.6
C600	0.03	58	2	3	4.4
C600	0.10	73	5	5	5.2

* C80=Rapidase C80; CL2000=Maxazyme CL2000; C600=Rapidase C600.

** expressed as percentage of fresh weight of chicory roots.

Rapidase C80 and Maxazym CL2000 chicory roots could be liquefied, however the amount of Rapidase C80 was still too high for recovery of inulin. Rapidase C600 gave a 73% recovery of total sugars, and a low amount of reducing sugars. This indicates that no inulin has been hydrolysed.

In order to confirm that inulinase activity of Rapidase C600 can be neglected, pure inulin was used as the substrate. Inulinase activity of Rapidase C600 was compared with commercial inulinase. Incubation of inulin with Rapidase C600 for 4 h showed no release of fructose, glucose, or saccharose.
Rapidase C600 was therefore used in all further experiments to investigate the release of inulin from chicory roots during enzymatic liquefaction.

7.2.2 *Release of inulin*

The release of inulin during enzymatic liquefaction of chicory roots has been studied with a commercial enzyme preparation, containing pectinases and cellulases. During 24 h of liquefaction, samples were taken and analysed for fructose, glucose, saccharose and inulin content by HPLC. The control was the same mixture without the addition of enzymes.
In the control mixture no increase is seen of the carbohydrates analysed, contrary to the liquefaction mixture. So no (endogenous) enzyme activity was found.
During liquefaction the glucose content of the supernatant increased due to hydrolysis of cellulose (Fig. 7.1).
The fructose content remained constant up to 5 to 8 h incubation time with commercial enzymes (0.1 resp. 0.01%) and then increased somewhat. Probably the (endo-) inulinase activity of Rapidase C600 is rather low, so that the effect of this enzyme (increase of fructose) is only seen after prolonged incubation (>4 h). No inulinase activity of Rapidase C600 was detected during incubation for 4 h at 40 °C at pH 4.5 when pure inulin was used as substrate. This was not investigated further.
Hydrolysis of inulin might also be due to endogenous inulinase activity of the chicory roots. Singh and co-workers (1971a, b) investigated fructose and inulin (fructosan) metabolism, and found that pH 5.6 and 37 °C were optimum

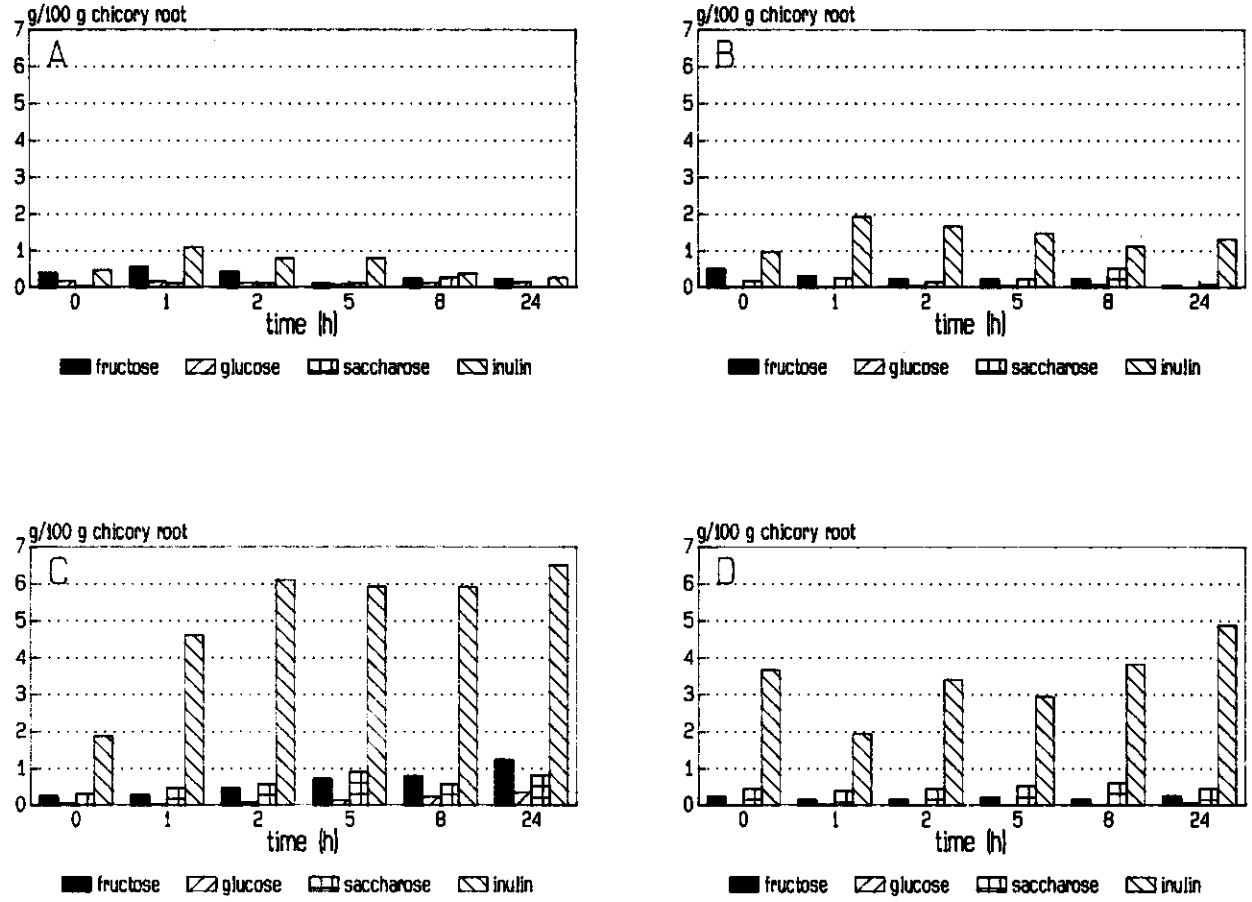


Fig. 7.1 Distribution of inulin and sugars between residue and supernatant during enzymatic liquefaction (A resp. C) with Rapidase C600 (0.1%) and control (B resp. D).

for fructosyl transferase, responsible for the synthesis of inulin. In later stages of the growth of chicory root, the enzyme fructosan hydrolase (inulinase) is responsible for the hydrolysis of fructosan (Gupta et al., 1985). No optimum conditions for the latter enzyme were reported. Probably fructosan hydrolase has the same optimum pH and temperature as has fructosyl transferase. If this is true then the pH during enzymatic liquefaction is too low for endogenous inulinase activity, however, the temperature used is almost optimal for this endogenous enzyme. Thus hydrolysis of inulin during enzymatic liquefaction of chicory roots by endogenous enzymes is not expected. As already mentioned before no inulinase activity was found in the control mixture, because no increase of fructose was seen.
Fig. 7.1 shows also that the supernatant from the enzymatic liquefaction contained almost all inulin originating from the chicory roots. Almost no inulin was found in the residue. However, the residue from the control liquefaction (without commercial enzymes) contained a relatively large amount of inulin. During the control liquefaction the chicory root cell walls are not hydrolysed and thus inulin will not pass into the liquid phase. An incomplete extraction is seen.

Optimum release of inulin is obtained after 2 h incubation with Rapidase C600 (Fig. 7.2). Incubation of the chicory roots with 0.1 or 0.01% Rapidase C600 gave no difference in release of inulin, only the time scale was different: hydrolysis of the cell wall components is more rapid when a higher amount of enzyme preparation is added to the root suspension. Thus optimum release of inulin is obtained after 2 h (Netjes, 1985).
Recovery of inulin in the control is approximately 100%, and for enzyme treated chicory roots between 100-150%. This great variation might be due to problems with inulin determination. Leclercq & Hageman (1985) showed that inulin can be determined by HPLC. However, only pure inulin could be detected; smaller heterogenous oligo-saccharides did not separate well on the column used, and one sugar gave double peaks. Retention times of sugars with the same DP were different.
Inulin content was therefore measured indirectly by determining fructose, glucose, and saccharose content before and after incubation with inulinase.

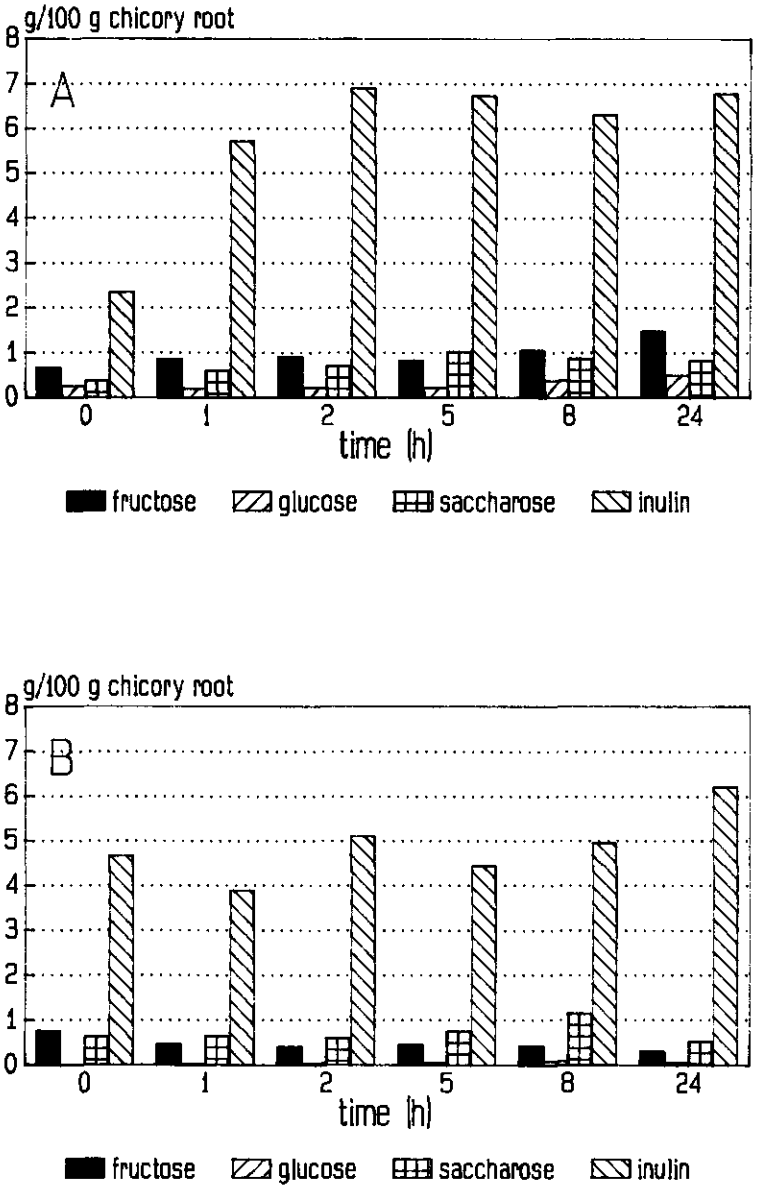


Fig. 7.2 Release of inulin and sugars during enzymatic liquefaction of chicory roots (A) and control mixture (B).

7.3 Conclusions

Inulin can be released by enzymatic liquefaction of chicory roots with a recovery of approximately 100%. The results of the liquefaction corresponds with repeated batch-wise liquid-solid extraction of the roots with hot water. The higher yield obtained during this liquefaction may thus be ascribed to the inefficiency of the incomplete one-step batch extraction of the untreated control, with which the liquefaction mixture was compared.
Incubation with Rapidase C600 (0.1%) has to be terminated within 5 h due to hydrolysis of inulin. No endogenous inulinase activity was found during incubation of the roots, which could be responsible for this hydrolysis.
No difference was seen between enzymatic treatment of the roots at 0.1 and 0.01% enzyme concentration. At low concentration only the hydrolysis was more lengthy.
Isolation of inulin using enzymatic liquefaction of chicory roots is warranted only in combination with the isolation of other interesting compounds, such as bitter sesquiterpene lactones, or for the production of bulk chemicals such as ethanol.

7.4 Experimental section

7.4.1 *Materials*

Chicory roots were obtained from a grower and stored one week at 1 °C before they were cut into small pieces (about 4 mm^3) under liquid nitrogen, and frozen at -60 °C before use.
Rapidase C80 (pectinases), Rapidase C600 (pectinases and cellulases), and Maxazym CL2000 (cellulases) were obtained from Gist-brocades, Delft, the Netherlands. Novozym 230, an inulinase preparation was obtained from NOVO industries, Copenhagen, Denmark. The inulinase activity according to the supplier was 3000 units/g.
Inulin was purchased from BDH Ltd (United Kingdom).

7.4.2 *Liquefaction of chicory roots*

Portions of frozen roots (10 g) were suspended in 0.1 M sodium acetate buffer (10 ml, pH 4) and incubated at 40 °C for 24 h under continuous stirring with 0.025 g Rapidase C80, 0.02 g Maxazym CL2000, 0.003 and 0.01 g Rapidase C600 , and 0.02 + 0.02 g and 0.025 + 0.02 g Rapidase C80 + / Maxazym CL2000 respectively. The enzymes were added to the suspension as dry powders. Samples were centrifuged (3000 g, 30 min) after incubation. The supernatant was analysed for reducing sugars using the Nelson-Somogyi test as modified by Spiro (1966). Total sugars were measured using the method according to Dubois et al. (1956). Fructose and glucose were both used for calibration.
The glucose, fructose, saccharose, and oligosaccharides content of the samples were determined with HPLC. An Aminex HPX-87 Pb column (300 x 7.8 mm; Bio-Rad) kept at 90 °C was used connected in line with a guard column filled with 35% AG50-WX4 (minus 400 mesh, H^+-form) and 65% AG3-X4A (200-400 mesh, OH^--form) both from Bio-Rad. Eluent was double distilled water degassed with helium. An RI detector was used (40 °C). The flow rate was 0.5 ml/min.

The release of inulin and sugars during enzymatic liquefaction of chicory roots was followed as function of time, as described in Section 5.3.3 for bitter compounds. The roots were incubated with Rapidase C600 (0.01 or 0.1%), which was added as dry powder to the chicory roots suspension. Samples were removed after 0, 1, 2, 5, 8, and 24 h. They were centrifuged for 30 min at 10000 g to obtain the residue and supernatant. Both were analysed for inulin, fructose, glucose, and saccharose. The control was the same mixture without added enzymes.

7.4.3 *Inulinase activity*

Inulinase activity of Rapidase C600 was compared with that of Novozym 230 by incubating pure inulin (100 ml of 5% solution) at 40 °C for 4 h at pH 4.5 with 0.05 g of Rapidase C600 or with 50 units of commercial inulinase.
The enzyme reaction was stopped by adding MeOH (4 ml of MeOH to 1 ml of inulin solution). The sample was filtered (0.45 μm filter), and analysed for fructose, glucose, and saccharose as described in Section 7.4.2.

7.4.4 *Analysis*

Fresh chicory roots, and residues and supernatants obtained during enzymatic and control liquefaction were analysed for fructose, glucose, and saccharose by HPLC as described in Section 7.4.2. The roots and residues (both 1.5 g) were extracted twice with water (5 ml, 70 °C). The two extracts were combined and the mixture was diluted with MeOH (4 ml of MeOH to 1 ml of total extract), filtered (0.45 μm filter), and injected into the liquid chromatograph. Supernatant was diluted with MeOH (as described above) before analysis.

The inulin content was measured indirectly after incubation with inulinase (Novozym 230). Aqueous extracts from chicory root and the residues, as well as the obtained supernatants, were incubated with inulinase. Inulinase (25 μl) was added to 1 ml of extract or supernatant, the pH adjusted to 4.5 with 0.1 N HCl and incubated (60 °C) for 3 h. Addition of MeOH (see above) was used to terminate the reaction. The samples were analysed for fructose, glucose, and saccharose as described in Section 7.4.2. The inulin content was calculated from the sugar content before and after incubation with inulinase.

CHAPTER 8

SENSORY ANALYSIS OF CHICORY ROOTS

8.1 Introduction

The first attempts to evaluate bitterness of lactucin and lactucopicrin were carried out with fresh and dried milky juice of *L. virosa* by Schenck & Graf (1939a, b). They found that lactucarium only contained the bitter compounds Lc and Lp. With this material they determined the lowest concentration at which Lc and Lp were perceived as bitter: about 2 and 1.6 ppm respectively (Schenck & Graf, 1939a).

A relation was also found between bitterness perception of lactucarium and composition of the soil in which *L. virosa* was grown (Schenck & Graf, 1939b). However, only the total bitterness of lactucarium was determined, and not that of the individual components. A more bitter lactucarium is obtained with an acid soil containing less calcium.

Dolezal (1976) investigated the influence of forcing methods on the yield of chicory heads and on the content of bitter principles in the roots as well as heads. Bitter compounds were analysed after extraction of the sample with acid methanol and reaction with KCN using fluorescence spectroscopy. Chicory heads proved to have a maximum of bitter compounds after forcing in synthetic foam flakes without any cover compared with forcing in soil with or without cover. In the roots no difference in bitterness was detected between the various forcing methods. However, the method of analysis for bitter constituents as used by Dolezal (1976) is inaccurate, because all compounds reacting with KCN are taken into account, including the compounds which are not bitter.

Kuusi & Autio (1985) investigated total bitterness of extracts of chicory heads and roots obtained by extraction with water. Kuusi & Autio (1985) compared bitterness of dandelion (*Taraxacum* sp.) with that of chicory. In dandelion extract they found glucosides of sesquiterpene lactones with a

germacrane skeleton, and also p-hydroxyphenyl acetic acid, which is a part of Lp, causing bitterness (Kuusi et al., 1985).

Voirol et al. (1987) compared total bitterness of an aqueous chicory root extract with that obtained from the leaves. The leaves appeared to be more bitter than the roots and bitterness persistency lasted longer for leaves than for chicory roots. They did not analyse chemically their extracts for bitter compounds. It is most likely (see Chapter 4 and 5) that the water extracts contained only precursor compounds (i.e. glycosides) and hardly any Lc and Lp. However, Voirol et al. (1987) presumed that these last two compounds are present in the extracts.

Dirinck et al. (1985), Price et al. (1990) and Van der Meer et al. (1985) tried to relate bitterness of chicory heads with chemical analysis of the bitter compounds. Dirinck et al. (1985) found a relationship between bitterness of fresh chicory heads and the amount of lactucin in chicory heads as measured by HPLC. However, their correlation coefficient is rather low ($r=0.69$).

Price et al. (1990) found a relationship between bound lactucin (lactucin glycoside) obtained after MeOH extraction of the chicory heads by boiling under reflux, and bitterness of fresh chicory heads ($r=0.80$). No relationship was found between the bitter score and the amount of 8dLc, Lp and their glycosides in chicory leaves. However, no evidence is given that the glycoside of Lc (picriside A, Fig. 6.2) was identified in the analysed samples. The water soluble components of the extracts were assumed to be the glycosides of Lc, 8dLc, and Lp. After treatment with a cellulase only these three sesquiterpene lactones were determined. As was seen in Chapter 4 many precursors are present in an MeOH extract of chicory roots. Various compounds from such an extract might be converted to Lc or other sesquiterpene lactones during incubation with cellulase.

It is also possible that by tasting a fresh sample of chicory in the mouth presursors are hydrolysed to their aglycons by endogenous chicory enzymes, which might both influence the results of Dirinck et al. (1985) and Price et al. (1990). Only three sesquiterpene lactones (Lc, 8dLc, Lp) were investigated. Price et al. (1990) could have known that chicory also contained other sesquiterpene lactones, such as dihydro equivalents (Seto et al., 1988), which were not taken into account. Pure compounds were not tested.

Van der Meer et al. (1985) related bitter taste of cooked chicory heads from various varieties with the respective extracts of uncooked samples. The extracts were analysed according to the rather unspecific fluorescence method as described by Dolezal (1976) after the reaction of the chicory extract with KCN. They found high correlation between bitterness score and chemical analysis (r=0.78-0.91). However, with their analytical method it is not possible to detect 8dLc (Van der Meer et al., 1985) and with the extraction method used (extraction with MeOH) a large amount of glycosides will be extracted and a low amount of bitter sesquiterpene lactones (see Chapter 4). It is uncommon to compare the chemical analysis of untreated chicory with the sensory analysis of a cooked equivalent. Also the influence of cooking on bitter principles of chicory has not been investigated, neither as pure compounds nor the effect on extraction efficiency and profile.

No publication is known in which the bitterness of pure, individual sesquiterpene lactones has been investigated and compared with for instance quinine. However, as can be seen from Fig. 4.1 B chicory root extract contains many compounds of which only few have been identified. We extracted and isolated six known sesquiterpene lactones (Lc, Lp, 8dLc, and their dihydro analogues), and determined their threshold value.
Further we investigated the possibility to use chicory root extract as flavouring material in soft drinks, especially tonic water, bitter orange, and bitter lemon, which usually contain quinine as bitter principle. The influence of daylight and pasteurization on bitter taste of bitter orange flavoured with chicory root extract or quinine as bitter constituent was studied. The effect of enzymatic treatment of chicory root extract on bitter taste was investigated.

8.2 Results and discussion

8.2.1 *Threshold values of sesquiterpene lactones*

Threshold values of pure Lc, Lp, 8dLc, dHLc, dHLp, dH8dLc were measured as described by Van Beek et al. (1990). In Table 8.1 threshold values of these compounds are summarized.

Table 8.1 Threshold values for the six sesquiterpene lactones and quinine hydrochloride (in ppm) (after Van Beek et al., 1990).

lactucin (Lc)	1.7
8-deoxylactucin (8dLc)	1.1
lactucopicrin (Lp)	0.5
11(S),13-dihydrolactucin (dHLc)	1.4
11(S),13-dihydro-8-deoxylactucin (dH8dLc)	1.1
11(S),13-dihydrolactucopicrin (dHLp)	0.2
quinine hydrochloride	1.6

Four sesquiterpene lactones, Lc, dHLc, 8dLc and dH8dLc, have threshold values similar to that of quinine hydrochloride. The other two, Lp and dHLp, are more bitter than quinine.

Schenck & Graf (1939a) have found a threshold value for lactucin and lactucopicrin from lactucarium of respectively 2 and 1.6 ppm. We found for lactucin a similar value, but the value for Lp was much lower.

Probably the preparation containing Lp used by Schenck & Graf (1939a) was not pure as was assumed. It is likely that is was contaminated with Lc and/or 8dLc.

In Chapter 3 it was predicted that lactucopicrin should be the most bitter compound of the three sesquiterpene lactones isolated from chicory roots, as a consequence of its structure, and that lactucin should be the least bitter compound. This has been corroborated by the afore mentioned experiment.

The same order of bitterness intensity is found also with the dihydro sesquiterpene lactones. Reduction of the exocyclic methylene group of the α,β-unsaturated lactone ring enhances bitterness somewhat. This can only be explained by increase of hydrophobicity of the molecules (see Chapter 3).

8.2.2 *Sensory analysis of soft drinks*

In the first experiment three types of soft drinks were prepared, i.e. tonic

Table 8.2. Composition of soft drinks (g/l).

	tonic	bitter orange	bitter lemon
fructose	23	21	0
glucose	37	22	50
sucrose	21	61	62
citric acid	3.6	0	0
Na-benzoate	0.5	0.5	0.5
orange juice*	0	100	0
lemon juice*	0	0	48
lemon extract*	0	0	40
quinine sulphate**	30	20	30
or			
chicory extract*	12	20	24

* *ml/l*
** *mg/l*

water, bitter orange, and bitter lemon with chicory root extract as the bitter principle. They were compared to identical controls, made with quinine sulphate. The composition of these drinks, summarized in Table 8.2, was determined after chemical analysis of commercial samples.

A higher concentration of chicory root extract was necessary in bitter orange lemonade and bitter lemon, since the bitter taste was masked by other flavour ingredients in the above mentioned beverages.

Bitterness of chicory root extract as flavouring material in tonic water was perceived to be different from that of quinine. About 37% of the panellists judged the beverage with chicory root extract as the most bitter, and 45% the beverage with quinine; the other panellists (18%) were not able to distinguish the beverage with chicory root extract from its reference with

quinine.
The second part of this experiment was a prefence test to establish the degree of liking for bitter orange or bitter lemon containing quinine or chicory root extract. No preference was given to quinine or to chicory root extract in bitter orange, contrary to bitter lemon in which quinine as bitter principle was most preferred ($p < 0.05$).
Quinine is an accepted and well known bitter constituent in soft drinks. Panellists are not familiar with the taste of chicory root extract as bitter principle in soft drinks. They may have rejected the beverage with this ingredient associating it with an off-taste.

In the second experiment the effect of pasteurization and storage in daylight or darkness on the bitter taste of bitter orange lemonade was measured. This beverage contained chicory root extract or quinine as bitter constituent (see Table 8.2 for the composition).
In the bitter orange lemonade containing chicory root extract a small difference in bitter score was seen between the pasteurized sample stored in darkness, and the untreated control (Table 8.3). Pasteurization had a small positive effect on the bitter taste. Storage in daylight of the pasteurized beverage decreased the bitter intensity slightly. However, the differences

Table 8.3 Influence of pasteurization and storage conditions on bitter score of bitter orange flavoured with quinine or chicory root extract (n=19).

	chicory root extract		quinine	
	mean	s.d.	mean	s.d.
untreated control, dark	57.1	8.3	14.3	8.8
pasteurization, dark	65.2	6.9	15.9	8.2
pasteurization, daylight	58.4	11.4	2.9	3.2

between these three samples are not significant ($p < 0.05$). Pasteurization of bitter orange containing quinine had no effect on the bitter taste, only storage of the beverage in daylight affected the bitterness which is significant ($p < 0.05$). This is in agreement with Sulser & Mändli (1987), who found in tonic water a practically complete degradation of quinine after a few hours when tonic was exposed to sunlight.

The bitter taste of the bitter oranges containing chicory root extract was perceived more intense when compared to the bitter oranges containing quinine despite the standardization of the used extract. After preparation of this extract, it was analysed, and standardized for the amount of lactucin as was the extract used in the first experiment. The influence of season, and variety when related to the bitter taste of chicory root extract is unknown, which both might have altered total bitterness. The extract was not standardized for the other bitter constituents.

8.2.3 *Bitterness of glycosides*

To investigate the influence of sesquiterpene lactone glycosides on bitterness of chicory root extract, the extract was first treated with Hemi II before sensory analysis. The experiment was carried out twice, because after the first experiment (10 panellists) no judgement could be given on whether the enzyme treatment of the chicory root extract gave rise to a more bitter taste.

In the first experiment 6 judges (n=10) could discriminate between the samples. All found the incubated extract the most bitter.

In the second experiment 17 panellists (n=36) could detect correctly the two pairs from four submitted samples. They too judged that the treated samples with Hemi II had the highest bitter intensity suggesting that the glycosides are less bitter when compared with their aglycons. However, the other 19 panellists could not detect any differences between the tasted samples.

From the above results it is not possible to conclude that precursors are less bitter than their aglycons. The influence of sugars, released by the exogenous enzymes, on the bitter taste is also not known. No pure compounds were evaluated, so the interpretation of the above results has to be carried out carefully.

8.3 Conclusions

The threshold values found for the six sesquiterpene lactones investigated (Lc, 8dLc, Lp, and their dihydro derivatives) depend to a great extent on the substituent at C-8. Lactucopicrin and its dihydro equivalent are more bitter than the other four compounds (about three to eight times). This is in agreement with the hypothesis formulated in Chapter 3.

Bitter orange, containing chicory root extract, does not lose its bitter taste upon storage in daylight. However, pure lactucin reacts with water under influence of daylight, which has been reported independently by Schenck et al. (1964), Leclercq et al. (1988), Sessink (1988), and Van Leeuwen (1989).
Schenck et al. (1964) proposed a mechanism for the addition of water to lactucin under the influence of daylight (Fig. 8.1), by which the molecule becomes more polar.

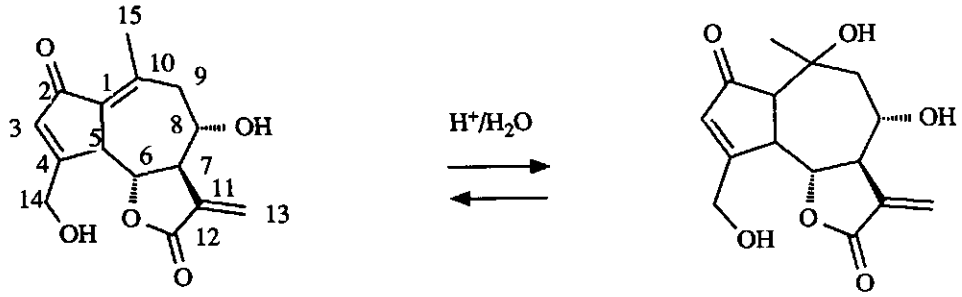


Fig. 8.1 Addition reaction of water to lactucin as proposed by Schenck et al. (1964).

The addition of water at C-10 is favoured above that of C-1 (also proposed by Schenck et al., 1964) for resonance reasons. The carbonyl group at C-2 is strongly polarized, with the electrons shifted toward the more electronegative oxygen atom. The carbonyl carbon is therefore electron deficient. This can be neutralized by the electrons from the C=C bonds

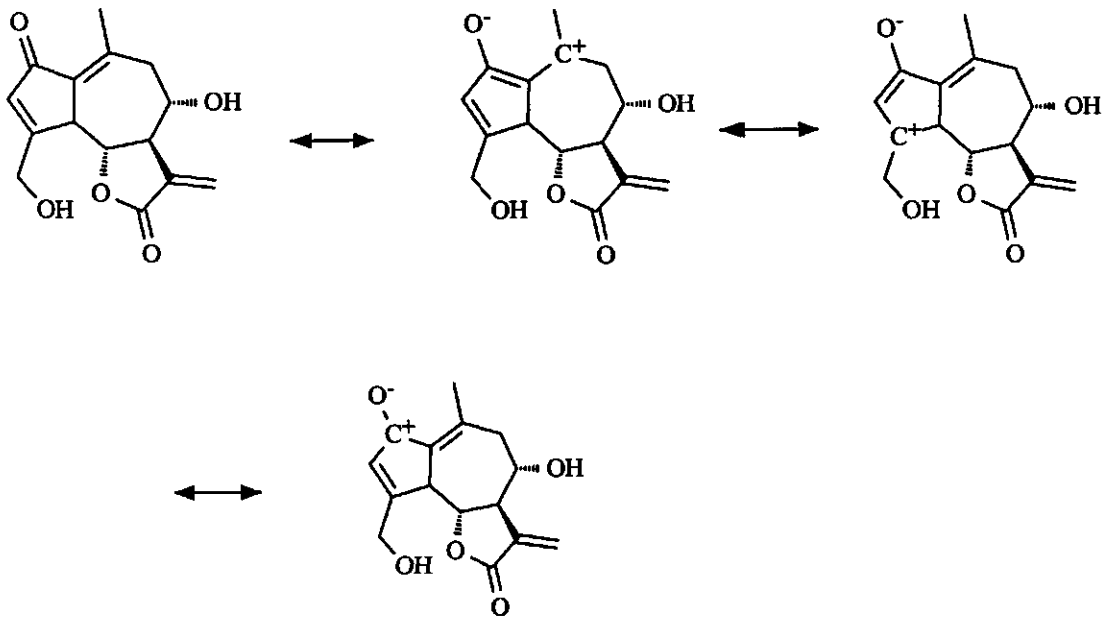


Fig. 8.2 Resonance structures of lactucin.

between C-3 and C-4, and between C-10 and C-1. The carbon atoms at C-4 and C-10 are thus slightly positively charged (Fig. 8.2). Addition of water at C-4 and C-2 next to addition at C-10 is possible (Fig. 8.3; Fig. 8.4), but these products have not been described in literature.

However, another reaction may also occur (Fig. 8.5). As a result of a photochemical reaction the double bond between C-10 and C-1 in the lactucin molecule is out of conjugation, and has been moved to C-10 and C-15 as described by Pfennig-Yeh according to Van Leeuwen (1989). The polarity of the solvent influences which type of reaction occurs (Van Leeuwen, 1989). In general a lot of energy is needed for a molecule to move out of conjugation. This mechanism is therefore not expected in the absence of (day)light.

According to the theories on bitterness, the newly formed compounds following addition of water should taste less bitter.

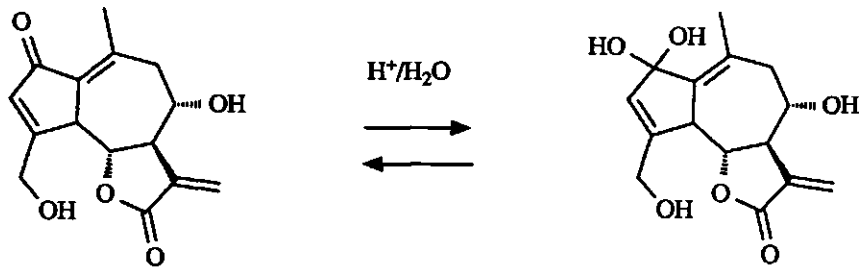


Fig. 8.3 Addition reaction of water to lactucin at C-2.

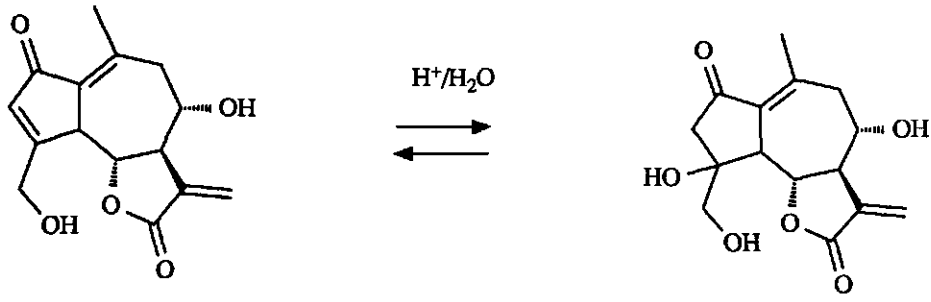


Fig. 8.4 Addition reaction of water to lactucin at C-4.

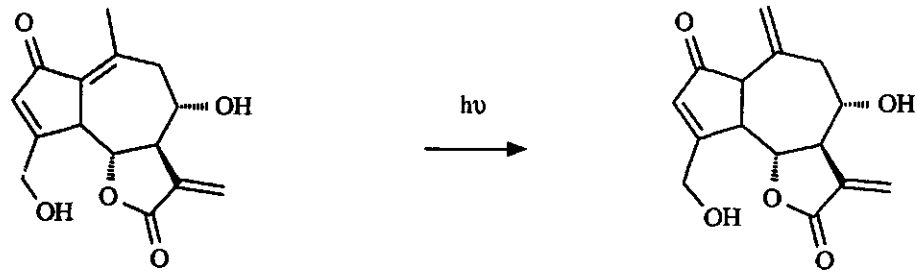


Fig. 8.5 Photochemical reaction of lactucin.

The photochemical reaction product of lactucin, which is out of conjugation, is expected to be equally bitter to Lc.
Probably this reaction with rearrangement of the double bond is favoured in bitter orange when stored in daylight, because equal bitterness is obtained when compared with bitter orange stored in dark. A stabilization effect of other ingredients during irradiation may also occur. This was not investigated further.

Quinine can be replaced as a flavouring material in soft drinks by chicory root extract, however the panel was not familiar with its bitter note, especially in bitter lemon. Probably this is also true for other bitter tasting compounds. Busch-Stockfisch & Domke (1991) reported that the bitter taste of amarogentin, a bitter tasting component of gentian root, was not identical to that of quinine, and that sucrose was necessary for obtaining a well-rounded bitter taste as well as for masking its off-taste. Bitterness of chicory root extract and that of quinine was also perceived as different.

Taste thresholds for bitter compounds show a Gaussian distribution, but for some bitter principles they are bimodal (see Chapter 3). Probably the population can be divided into tasters, which do recognize chicory bitterness, and tasters, which are not sensitive for the bitter taste of a chicory root extract. The same phenomenon may be true for the bitterness of the sesquiterpene lactones and their glycosides. About half of the panellists judged the glycosides to be equally bitter as their aglycons, the other part judged the sesquiterpene lactones as most bitter.
However, the interpretation of the above results has to be carried out very carefully, since no pure compounds were used. A complex mixture of precusors and their aglycons were tasted before and after enzymatic treatment. Not known are the effects of the release of the sugars from the glycosides on the bitter taste of the aglycons, i.e. whether there are synergistic or antagonistic effects of the various ingredients of the chicory root extract.

8.4 Experimental section

8.4.1 *Determination of threshold value*

Threshold determinations of six pure sesquiterpene lactones were determined by triangle testing. A professional panel of 16 housewives (40-55 years old) employed by Quest International was asked to select the one cup, out of three cups, that contained a stimulant other than water. Evaluations were conducted at room temperature. Samples were presented as 50 ml aqueous solutions in plastic serving cups.

8.4.2 *Sensory analysis of soft drinks*

Chicory root extract was prepared as described in section 5.3.2. The extract was analysed and standardized for the amount of Lc (30 mg/100 ml extract; 20 ml of extract (6 mg of Lc) was judged equally bitter as 20 mg quinine sulphate per l bitter orange). This extract was used as a bitter ingredient in three types of soft drinks: tonic water, bitter orange, and bitter lemon. They were compared with the same type of beverage containing quinine sulphate. The composition of the beverages is summarized in Table 8.2.
Lemon extract was prepared by steam distillation of lemon peel (340 ml extract was obtained from the peel of one lemon). Lemon juice was freshly pressed prior to use. Orange juice was bought in the supermarket.

In the first experiment a consumer panel consisting of selected housewives (n=102) was asked to identify the most bitter beverage out of two, thus comparing the quinine flavoured beverage with the chicory flavoured beverage. Only one type of beverage (for instance tonic water) was judged each time. Samples were presented as 50 ml solutions in plastic serving cups at room temperature.

In the second experiment a panel consisting of students from the Agricultural University, Wageningen (n=17) was asked to rank 6 samples of bitter orange lemonade. The beverages were prepared with chicory root extract or quinine sulphate as bitter ingredient. The composition of the beverages is summarized

in Table 8.2. The influence of different process conditions on the bitterness of the beverages was investigated. A part of the bitter orange was pasteurized (80 °C, 30 min), and divided into two portions: one was stored in daylight (june 1990), the other part in darkness at 5 °C, both for 3 days. The non-pasteurized bitter orange samples (control) were stored in dark at 5 °C for 3 days prior to use. The six samples were presented as 20 ml solutions in plastic serving cups at room temperature, and were presented simultaneously in random order. The students were selected for their ability to perceive a bitter taste.

8.4.3 *Bitterness of glycosides*

Chicory root extract was prepared as described in section 5.3.2. The thus obtained aqueous extract was divided in two portions, one of which was incubated with 0.05% enzyme preparation (Hemi II, Gist-brocades, the Netherlands) for 26 h at 30 °C under continuous stirring. Inactivation of the enzymes was by boiling. The extracts were diluted 5 times with tap water before further analysis.
The treated and untreated chicory root extract was tasted by a student panel (n=36), which was selected for their ability to taste bitter. Samples were presented as 50 ml aqueous solutions in plastic serving cups at room temperature. The panel was asked to identify two pairs out of four samples, and to state which pair had the highest bitter intensity.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUDING REMARKS

9.1 Enzymatic liquefaction

Isolation of substances from plant material is usually carried out with a solvent. Complete extraction of both bitter sesquiterpene lactones and inulin from chicory roots in one step is not possible when extraction is performed with one solvent.
Inulin is soluble in (hot) water. It is extracted from the roots by liquid-solid extraction. Countercurrent diffusion may also be used in place of batchwise extraction (Fleming & Groot Wassink, 1979). The sesquiterpene lactones are rather apolar and will thus remain in the pulp fraction. Extraction with more apolar solvents give only the bitter substances as solvent solubles, and inulin will remain in the pulp.

According to our results a one step process is only possible when enzymes are used to degrade chicory root cell wall polysaccharides, in order to solubilize inulin as well as the bitter substances. An almost complete liquefaction can be obtained when pectinases and cellulases are involved (Pilnik & Rombouts, 1979). However, such a process is rather unusual for the isolation of flavour compounds.
With enzymatic liquefaction of chicory roots not only inulin and bitter compounds will pass into the liquid phase, but also a higher yield is expected, especially for the bitter principles, compared to solvent extraction.

The extraction of the roots with different solvents is investigated to determine the effect of solvents on extraction efficiency and yield, for analytical purposes (development of an HPLC method), and for comparison with literature data.

Of all solvents evaluated for extraction of bitter compounds from chicory roots EtOAc is the best one, giving a simple procedure and reproducible extracts. Chicory root extracts obtained from more polar solvents, such as water and MeOH, consist of mainly polar compounds and less Lc, 8dLc, and Lp. These polar compounds elute at the beginning of the HPLC chromatogram (see Chapter 4). Some of them are expected to be glycosides of the sesquiterpene lactones investigated. Seto et al. (1988) extracted chicory roots with MeOH and found many glycosides in the solvent solubles.

Storage conditions, the degree of milling, or freezing, all influence the total yield and extraction efficiency of the sesquiterpene lactones investigated (Chapter 4). Storage at low temperature (1 °, -30 °C) gives a higher yield of especially the apolar compounds. Drying of the roots at 70 °C degrades bitter compounds almost completely. The same results have been described for the bitter substances from *Taraxacum*, which are glycosides of germacranolides (Kuusi et al., 1985).
The milling step is critical for obtaining a reproducible extract from chicory root. Endogenous enzymes may be active during this process, converting precursor compounds into other compounds. The yield of sesquiterpene lactones, the reproducibility of the extraction, and also the bitter tast of the extract are influenced in this way.
Freeze dried chicory root powder has first to be soaked in an excess of water to obtain high extraction efficiency with EtOAC as solvent. Water seems to play an important role during extraction. This might be due to a low solubility of carbohydrates in the apolar extraction solvent and may have been prevented the sesquiterpene lactones to come into contact with the solvent.

When chicory roots are liquefied enzymatically with commercial pectinases and cellulases more sesquiterpene lactones are isolated compared to roots which are extracted after milling and/or frozen storage (Chapter 5). Especially Lc, dHLc, 8dLc, and dH8dLc increased during enzyme treatment of the roots. This increase can be explained by:

i. Total solubilization of the chicory roots by exogenous enzyme treatment. Thus all compounds present in the roots can be detected in the liquid phase.

Extraction efficiency of the roots with a solvent will also depend on diffusion of the compounds through the cells, and on interactions of the solvent solubles with other cell components. These effects can be neglected after complete liquefaction.

ii. Conversion of precursors (glycosides) into their sesquiterpene lactone aglycons by added commercial enzyme preparations or by endogenous chicory enzymes or both. However, the optimum conditions for both enzyme systems are different: optimum pH and temperature are pH 4-4.5 and 40 °C, and pH 6 and 30 °C respectively. During enzymatic liquefaction of chicory roots, exogenous enzymes are responsible for the hydrolysis of precursors into the sesquiterpene lactones investigated. Chicory root enzymes are also able to catalyse the same type of reactions, but only at higher pH and at slightly lower temperature (Chapter 5, 6). Endogenous chicory enzymes play a role during processing and storage of the roots.
The hydrolysis of two precursors, cichorioside B and crepidiaside B (compound Q and compound T respectively), into dHLc and dH8dLc was found during incubation of chicory roots and chicory root extract both with commercial enzymes containing pectolytic and cellulolytic activity and with endogenous chicory enzymes. Incubation of a chicory root extract (inactive endogenous enzymes) with a commercial enzyme preparation shows only an increase in dHLc, contrary to the incubation with chicory root enzymes, where also an increase in Lc is seen. The glycoside of Lc, picriside A, is probably present in the extract, but is not hydrolysed by the enzymes from the commercial preparation.
The nature of compound N is still unknown. It is converted in cichorioside B during incubation with the used enzymes. There are indications are that compound N is a diglycoside of dHLc.

iii. Possible conversion of sesquiterpene lactones with a germacrane skeleton into sesquiterpene lactones with a guaiane skeleton. Transformation of compounds with a germacrane skeleton into compounds with guaiane skeleton has been described with endogenous chicory enzymes (Chapter 6). When chicory root extract is incubated with endogenous root enzymes for more than 24 h, only sesquiterpene lactones with a guaiane skeleton are found in the HPLC chromatogram.

Whether commercial enzyme preparations are able to convert a germocranolide into a guaianolide is still a subject of speculation. It is plausible that endogenous chicory enzymes are capable to perform this reaction. Both types of sesquiterpene lactones are found in the chicory root. More research is needed to evaluate commercial enzyme preparations as well as chicory enzymes for their ability to catalyse the above mentioned reactions and to evaluate above findings with pure compounds. The result might be valuable for new applications in the synthesis of terpenes.

Use of pectinases and cellulases for isolation of flavour compounds from plant material is not common (Chapter 1). Glucosidases have been described in literature for hydrolysing monoterpene glucosides through which volatile monoterpenes are released, in order to enhance the aroma of fruit juices and wines (Wilson et al., 1984; Voragen, 1989).
Similar effects may be true for other vegetables and plant materials subjected to enzymatic liquefaction. Endogenous enzymes may be active during this treatment. It can be stated that by enzymatic liquefaction more interesting compounds are released in the liquid phase (juice), and depending on conditions chosen more precursors are released and can be hydrolysed into their aglycons, thus changing flavour and taste of the juice. Also non-volatile taste components can be released. Jenniskens et al. (1991) studied the effect of liquefying enzymes on the aroma constituents of apple juice. Liquefaction caused an extra release of aroma compounds or precursors of flavour compounds from the pulp into the juice. To date no results are reported discussing vegetables in this context.

Inulin can be released during enzymatic liquefaction. When compared with hot water extraction, the yield of inulin is about the same. Enzymatic treatment in order to release inulin is only economically feasible when other interesting compounds can be released simultaneously (Chapter 7).

9.2 Sensory analysis

The six sesquiterpene lactones which were investigated, have been proven to taste bitter. Their threshold value varies depending on the group attached to

C-8. Lactucopicrin and 11(S),13-dihydro-lactucopicrin proved to be the most bitter compounds of the six lactones investigated, and lactucin and its dihydro derivative were the least bitter, but have about the same threshold value as quinine hydrochloride.

Sensory analysis of soft drinks prepared with chicory root extract as such, and after enzymatic treatment, suggested that glycosides of the sesquiterpene lactones are less bitter than their respective aglycons. However, an extract was tasted and not the pure, isolated compounds. It was difficult to obtain a chicory root extract with similar bitter intensity compared to a previous prepared extract made from another portion of chicory roots. Storage of the roots increased the amount of especially apolar compounds, and therefore its bitterness.
The effect of natural variation (season, variety) on the bitter taste of chicory root and its extract is not known.
Chicory extract contained cichorioside C, a germacranolide. Kuusi et al. (1985) isolated from *Taraxacum* bitter glucosides of sesquiterpene lactones with a germacrane skeleton. It might be expected that cichorioside C (as part of compound Q) has a bitter taste. However, the bitter intensity of this compound is not known, nor that of the other unknown components present in chicory root extract.
Eudesmanolides have been identified in *C. intybus and C. endivia*, a relative of chicory (Seto et al., 1988). This was not confirmed by other authors. In the chicory roots extracts described in this thesis no sesquiterpene lactones with an eudesmane skeleton were found. However, many compounds were not identified further. It is therefore conceivable that our chicory extract also contained eudesmanolides. Their possible influence on the bitter taste is not known, but some eudesmanolides are reported to taste bitter (Hänsel et al., 1980).

After incubation of chicory roots with endogenous enzymes only guaianolides are obtained. This may provide a possibility to prepare a chicory root extract of a more constant bitter quality. An extract which consists of only a few compounds is more easy to handle.
Depending on the final application, various chicory root extracts may be produced with different bitter intensities. The bitterness of the extract can

be influenced by choosing the desired condition: enzymatic liquefaction, treatment with endogenous chicory enzymes, or extraction with polar or apolar solvents.

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SUMMARY

Chicory (*Cichorium intybus* L.) is one of the many species of the family Compositae. Chicory has been cultivated for the production of leaves or chicons, which have been used as a vegetable since approximately 300 BC, and for its roots, which can be used as a coffee substitute after roasting.
Chicory leaves are appreciated for their slightly bitter taste. Two bitter compounds were known at the start of this project: lactucin (Lc) and lactucopicrin (Lp), both sesquiterpene lactones with a guaiane skeleton. These compounds are also present in the roots, which remain as a waste product after harvesting of the chicons. Chicory roots contain besides bitter substances also inulin, a linear β-(2-1) linked fructose polymer terminated by a sucrose unit residue and the main carbohydrate of the chicory plant.
In Chapter 2 all known constituents of chicory roots are discussed as well as the effect of roasting on these compounds. A survey is given of work carried out on the isolation and identification of bitter principles in Compositae, especially chicory. The aim of this project was to isolate the bitter constitutents and inulin in one step from waste chicory roots. A one step process is only possible when enzymatic liquefaction is applied. Both bitter compounds and inulin will then pass into the liquid phase. The obtained bitter, sweet liquid can be used as a raw material for soft drinks. Quinine eventually could be replaced by the bitter principles from chicory roots.

An isocratic HPLC method was developed for the analysis of the sesquiterpene lactones. Three components were identified in the chicory root extract: Lc, Lp and 8-deoxylactucin (8dLc). Various extraction solvents were tried for the isolation of the sesquiterpene lactones from chicory roots. Polar solvents gave many unknown polar compounds, which eluted at the beginning of the HPLC chromatogram. More apolar solvents gave the sesquiterpene lactones and hardly any of the polar components.
Storage of the roots and further processing, such as drying and milling, affects the amount of sesquiterpene lactones in the roots and thus the composition of the chicory extract (Chapter 4).

The release of bitter compounds and inulin has been studied during enzymatic

liquefaction of chicory roots with commercial pectinases and cellulases (Chapter 5 and 7). An increase was seen in the amount of Lc and 8dLc found in the liquid phase during enzymatic liquefaction. After improvement of the HPLC method (gradient elution instead of isocratic method) it was found that the increase of Lc and 8dLc was due to the increase of their dihydro derivatives, which eluted at the same place as Lc respectively 8dLc with the isocratic method.

Endogenous chicory root enzymes have also been studied in this context, because they have proven to be capable to release bitter components as well (Chapter 6). However, the optimum pH and temperature for the performance of endogenous chicory root enzymes are different from those of the commercial enzyme preparations tested, and may therefore not play a role in the release of sesquiterpene lactones during enzymatic liquefaction.

Cichorioside B (glycoside of 11(S),13-dihydro-lactucin), crepidiaside B (glycoside of 11(S),13-dihydro-8-deoxylactucin), cichorioside C (glycoside of a germacranolide), and 11(S),13-dihydrolactucopicrin were identified in chicory roots. Compound N could not be identified, but there are indications that this compound is a diglycoside of dHLc. The presence of the glycoside of Lc is plausible, but to date this compound was not extracted from the chicory roots.

The threshold value of six pure sesquiterpene lactones (Lc, Lp, 8dLc, dHLc, dHLp, dH8dLc) was determined (Chapter 8) and related to the theories on bitterness as discussed in Chapter 3.

The effect of processing and storage on the bitter taste of bitter orange lemonade was investigated. A comparison was made between quinine as bitter substance and chicory root extract as the bitter ingredient.

The storage in daylight of the bitter orange containing quinine caused a tremendous decrease of the bitterness of the beverage. No decrease in bitterness was seen in the beverage with chicory root extract. Pasteurization did not affect the bitter taste of bitter orange with chicory root extract.

The bitterness of the various chicory root extracts made for sensory analysis differed in bitter intensity in spite of standardisation of the Lc content.

Bitter intensities of chicory root extract before and after incubation with pectolytic and cellulolytic enzymes were determined. Thus the bitterness of the precursors was compared with that of the aglycons. However, no judgement could be given on whether the enzyme treatment of the chicory root extract

could be given on whether the enzyme treatment of the chicory root extract gave rise to a more bitter taste. About half of the panellists judged the extract with the glycosides more bitter than the extract with the aglycons, the other half could not taste any difference between these samples.

SAMENVATTING

Witlof (*Cichorium intybus* L.) is een van de vele soorten van de familie der Composieten. Als sinds de oudheid worden de bladeren als groente gegeten. De geroosterde wortels worden gebruikt als koffiesurrogaat. Dit gebruik stamt uit de 16e eeuw.
Witlof smaakt enigszins bitter. Deze bitterheid werd bij de start van dit project toegeschreven aan twee sesquiterpeen lactonen: lactucine en lactucopicrine. Deze twee verbindingen komen ook in de wortels voor.
Witlofwortels komen vrij na het oogsten van het lof. Ze worden als afvalproduct beschouwd. Behalve de bitterstoffen bevatten de wortels ook het polysaccharide inuline. Het doel van dit onderzoek was de isolatie van zowel inuline als de bitterstoffen uit de witlofwortels met behulp van enzymatische vervloeiing. Op deze manier zou een bitter, zoete stroop kunnen worden verkregen, die als grondstof kan dienen voor de bereiding van bittere dranken, zoals tonic. Tonic bevat nu kinine. Sommige mensen zijn gevoelig voor kinine en vertonen een allergische reactie als kinine in het dieet voorkomt.

In Hoofdstuk 2 worden alle tot nu toe geïdentificeerde inhoudstoffen van witlofwortels besproken. Het effect van roosteren op deze verbindingen is nagegaan. Ook is een overzicht opgenomen van publicaties over de isolatie en identificatie van bittere stoffen in Compositae, waarbij vooral de aandacht aan witlof wordt gegeven.
Het isoleren van zowel inuline als de bitterstoffen in één extractieprocedure is niet goed mogelijk. Inuline is oplosbaar in (heet) water, de bitterstoffen niet. De sesquiterpeen lactonen kunnen worden geëxtraheerd met een apolair oplosmiddel, maar dan blijft inuline in de restfractie achter. Als oplossing voor dit probleem is daarom gekozen voor een complete vervloeiing van de witlofwortels met behulp van pectolytische en cellulolytische enzymen. De celwanden worden daarbij afgebroken en de gehele celinhoud komt in de vloeibare fase terecht.

Als eerste werd de extractie en analyse van de bitterstoffen ter hand genomen (Hoofdstuk 4). Een isocratische HPLC methode werd ontwikkeld, waarmee de extracten van de witlofwortels werden geanalyseerd. In het witlofwortel

extract werden drie sesquiterpeen lactonen aangetoond: lactucine (Lc), lactucopicrine (Lp) en 8-deoxylactucine (8dLc).
Het soort oplosmiddel dat tijdens een extractie werd gebruikt bleek van grote invloed op de hoeveelheid geëxtraheerde sesquiterpeen lactonen. In een polair oplosmiddel kwamen voornamelijk polaire stoffen voor, eluerend in het begin van het HPLC chromatogram, en nauwelijks Lc, Lp en 8dLc. Werd een apolair solvent gebruikt dan werden vooral deze drie verbindingen in het extract aangetroffen en weinig polaire verbindingen. De precieze aard van deze polaire stoffen is onbekend, maar vermoedelijk betreft het glycosiden.
Ook de bewaarcondities beïnvloeden het extractierendement, evenals de bewerking (malen, drogen). De wat wisselende resultaten met betrekking tot de opbrengst aan bitterstoffen kon in de loop van het onderzoek worden toegeschreven aan de endogene enzymactiviteit van de witlofwortels zelf.

Het vrijkomen van bitterstoffen en inuline gedurende enzymatische vervloeiing is bestudeerd (Hoofdstuk 5 en 7). De hoeveelheid bitterstoffen bleek tijdens vervloeiing toe te nemen ten opzichte van een blanko vervloeiing (dezelfde condities werden aangehouden, alleen werd geen enzym toegevoegd). Met name de hoeveelheden Lc en 8dLc namen toe.
Na optimalisatie van de HPLC methode (een gradient elutie werd gebruikt in plaats van de isocratische methode) kon worden aangetoond dat de toename van Lc en 8dLc het gevolg was van de enzymatische hydrolyse van de glycosiden van 11(S),13-dihydro-lactucine (dHLc) en 11(S),13-dihydro-8-deoxylactucine (dH8dLc). Beide dihydro-derivaten vielen in het isocratische HPLC chromatogram samen met de pieken van Lc en 8dLc.
Endogene witlofenzymen bleken ook in staat om bovenstaande omzettingen te geven. Echter de optimale pH en temperatuur van deze enzymen verschilt van die van het gebruikte enzympreparaat tijdens vervloeiing. Gedurende de enzymatische vervloeiing met exogene enzympreparaten zijn de endogene enzymen waarschijnlijk niet actief.
In witlofwortels zijn aangetoond cichorioside B (glycoside van dHLc), crepidiaside B (glycoside van dH8dLc), cichorioside C (glycoside van een germacranolide) en 11(S), 13-dihydro-lactucopicrine (dHLp). De aanwezigheid van het het glycoside van Lc werd aannemelijk gemaakt, maar deze stof kon tot op heden niet worden geïsoleerd. Een andere verbinding, component N, werd tijdens de enzymatische vervloeiing omgezet in cichorioside B. Waarschijnlijk

gaat het hier om een diglycoside van dHLc, maar dit is niet nader onderzocht.

De drempelwaarde van zes sesquiterpeen lactonen (Lc, Lp, 8dLc, dHLc, dHLp, dH8dLc, uit witlofwortels geëxtraheerd en gezuiverd) is bepaald en vergeleken met die van kinine (Hoofdstuk 8). De uitkomst is gerelateerd aan de theorieën over bitterheid. Deze zijn in Hoofdstuk 3 besproken.
De bitterheid van een witlofwortel extract in tonic, bitter orange, en bitter lemon is vergeleken met die van kinine. De bitterheid van het extract werd anders waargenomen dan die van kinine. Ook bleek de bitterheid van de gemaakte extracten verschillend te zijn, ondanks de standaardisatie van het Lc-gehalte.
Het effect van pasteurisatie op de bitterheid van bitter orange waaraan witlofextract is toegevoegd en het effect van bewaren in daglicht werden onderzocht. Beide bewerkingen hebben weinig effect op de bitterheid van bitter orange. Dit in tegenstelling tot bitter orange, waaraan kinine als bitterstof was toegevoegd. Met name bewaren in daglicht had een negatief effect op de bitterheid van de drank.
De bitterheid van een witlofextract voor en na incubatie met pectolytische en cellulolytische enzymen werd bepaald om te onderzoeken of de glycosiden even bitter zijn als hun aglycon. Hierover kon geen uitspraak worden gedaan, omdat de bitterheid van het extract met glycosiden anders was dan die van het behandelde extract. Het experiment zal daarom moeten worden herhaald met zuivere glycosiden.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren in 1955 te Drachten. In 1972 behaalde zij het diploma Atheneum B aan het Drachtster Lyceum in Drachten. Na het behalen van de akte N XIX (1975) begon zij aan de studie Levensmiddelentechnologie aan de Landbouwhogeschool te Wageningen. Het doctoraal diploma werd in 1982 behaald. In dat jaar werd zij aangesteld als wetenschappelijk onderzoeker bij het toenmalige Sprenger Instituut te Wageningen, waar het onderzoek zoals beschreven in dit proefschrift, in nauwe samenwerking met de vakgroepen Levensmiddelenchemie en Organische Chemie, werd uitgevoerd.
Tegenwoordig is zij als onderzoeker werkzaam bij Quest International te Naarden.